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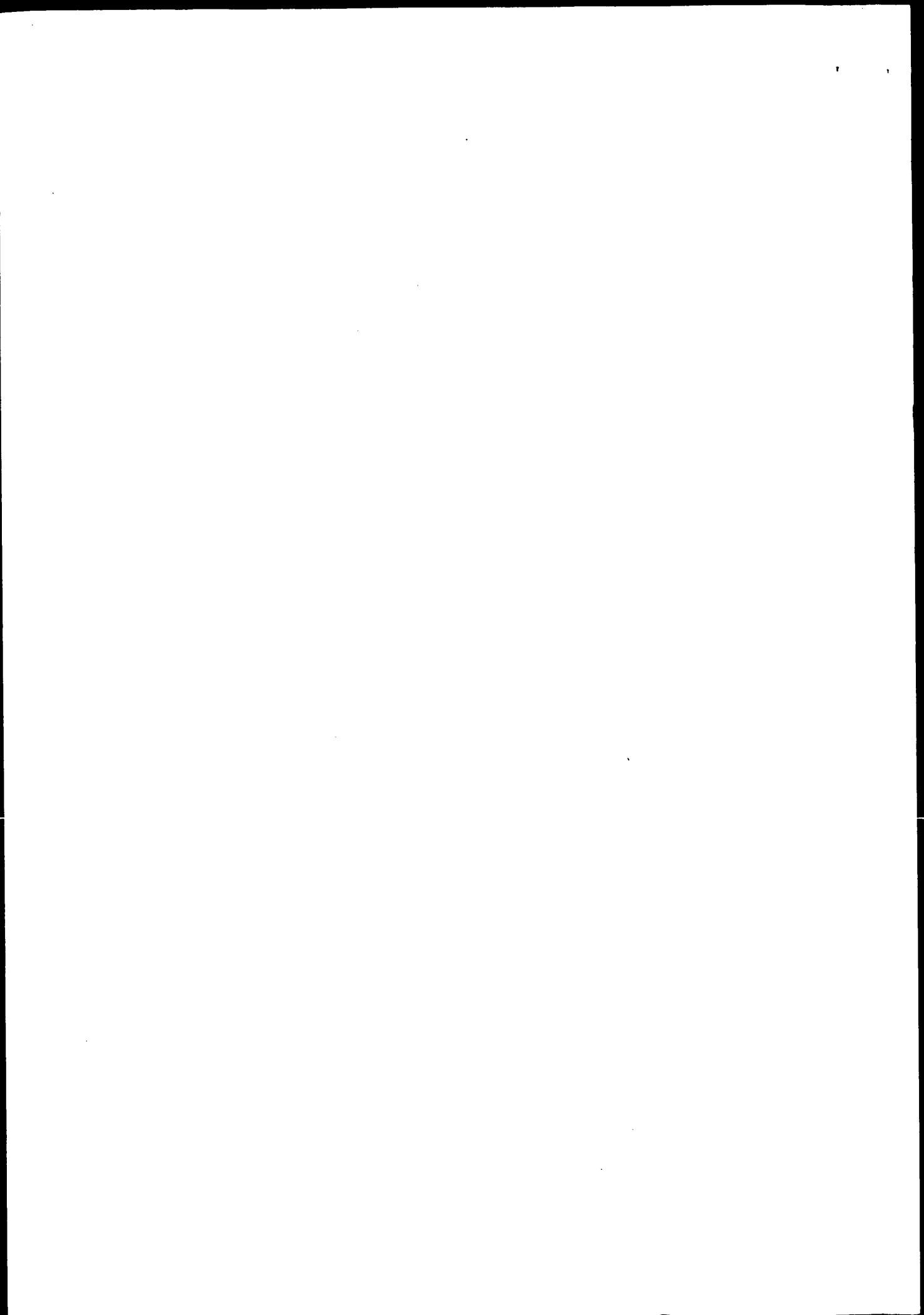3-6, Doshomachi 2-chome Chuo-ku
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㊴ Proteins and production thereof.

㊝ Disclosed are (1) a fused protein obtained by combining an antigen used for vaccine and a lymphokine by the application of gene engineering, (2) a recombinant DNA containing a nucleotide sequence coding for the above fused protein, (3) a transformant bearing the above recombinant DNA, (4) a method for producing the fused protein which comprises cultivating the above transformant, producing and accumulating the above fused protein in a culture, and collecting the fused protein, and (5) a hybrid protein obtained by chemically combining an antigen used for vaccine with a lymphokine. The resulting fused and hybrid proteins have strong immunogenicity.

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present invention;

Fig. 12 is a representation showing an amino acid sequence deduced from the nucleotide sequence shown in Fig. 11;

Fig. 13 is a schematic representation showing the construction of an expression plasmid for animal cells of the truncated gD gene of HSV-1;

Fig. 14 is a schematic representation showing the construction of an expression plasmid for animal cells of the fused protein gene according to the present invention;

Fig. 15 is a graph showing survival rates of mice to time after inoculation of HSV.

Figs. 16-1, 16-2, 16-3, 16-4, 16-5, 16-6 and 16-7 are schematic representations showing the construction of plasmids used in Reference Example 2;

Fig. 17 is a representation showing a nucleotide sequence and an amino acid sequence deduced from the nucleotide sequence of gpl gene inserted into the plasmid pUC18 in Reference Example 2;

Fig. 18 is a schematic representation showing the construction of an expression plasmid for animal cells of the fused protein gene according to the present invention;

Fig. 19 is a schematic representation showing the construction of an expression plasmid for animal cells of the fused protein gene according to the present invention;

Fig. 20 shows Western blotting analysis of the fused protein of the present invention;

Fig. 21 is a schematic representation showing the construction of an expression plasmid for animal cells of the fused protein gene according to the present invention; and

Fig. 22 is a schematic representation showing the construction of an expression plasmid for animal cells of the fused protein gene according to the present invention;

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Preferred lymphokines for use in the present invention include interleukin (hereinafter referred to as IL)-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, granular colony stimulating factor (G-CSF), granular macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF) and interferon-γ.

The antigens (proteins or polypeptides) used for vaccines in accordance with the present invention include antigens of viruses whose hosts are animals, such as antigens of herpesviruses including herpes simplex virus (HSV), varicella-zoster virus (VZV) and cytomegalovirus (CMV); antigens of retroviruses including human immunodeficiency virus (HIV) and adult human T cell leukemia virus (HTLV-I); antigens of hepadonaviruses including hepatitis B virus (HBV); antigens of togaviruses including non-A, non-B hepatitis viruses (HCV and HEV) and Japanese encephalitis virus; antigens of picornaviruses including hepatitis A virus (HAV); antigens of orthomyxoviruses including influenza virus; antigens of parvoviruses; antigens of papovaviruses; antigens of adennoviruses; antigens of poxviruses; antigens of reoviruses; antigens of paramyxoviruses; antigens of rhabdoviruses; antigens of arenaviruses; and antigens of coronaviruses; antigens of pathogenic protozoa such as a malarial antigen; and antigens of pathogenic bacteria such as a Bordetella pertussis antigen.

Examples of such antigens include surface antigen gD or gB of herpes simplex virus (HSV) type 1 or type 2, surface antigen gpl or gpIII of varicella-zoster virus (VZV), gag antigen or env antigen of human immunodeficiency virus (HIV), gag antigen or env antigen of adult human T cell leukemia virus (HTLV-I), C antigen, M antigen or E antigen of hepatitis C virus (HCV), and core antigen, surface antigen L protein, surface antigen M protein or surface antigen S protein of hepatitis B virus (HBV).

In some embodiments of the present invention, the antigen used for vaccine may be fused with the lymphokine through a linker. In other embodiments, a hybrid protein comprising an antigen used as a vaccine and a lymphokine is formed by chemical methods.

Linkers for use in the present invention comprise one amino acid residue or a peptide residue comprising 2 to about 30 amino acid residues (preferably one amino acid residue or a peptide residue comprising 2 to about 10 amino acid residues) selected from G, A, V, L, I, S, T, C, M, E, D, K, R, H, F, Y, W, P, N and Q.

As an example, a fused protein of an HSV surface protein which is an HSV antigen with IL-2 will hereinafter be described.

As the HSV surface protein, glycoproteins gD and gB lacking transmembrance domains are advantageously used.

The present invention particularly provides (1) fused protein (I) of glycoprotein gD lacking the transmembrane domain with IL-2, or fused protein (II) of glycoprotein gB lacking transmembrane domain with IL-2; (2) recombinant DNAs (III) and (IV) containing nucleotide sequences coding for fused proteins (I) and (II), respectively; (3) transformants bearing recombinant DNAs (III) or (IV), respectively; and (4) a

method for producing fused protein (I) or (II) which comprises cultivating the transformant bearing recombinant DNA (III) or (IV), producing and accumulating fused protein (I) or (II) in a culture, and collecting fused protein (I) or (II).

5   As surface protein genes of HSV, there can be used, for example, gD and gB genes of various HSV-1 strains such as HSV-1 strain Miyama. Examples of the gD genes include a gene having the amino acid sequence shown in Fig. 1 (surface protein gD of HSV-1 strain Miyama, Japanese Patent Application No. 63-180114/1988). The essential portion of this amino acid sequence is from Lys of No. 26 to Ala of No. 302. Examples of the DNAs containing the nucleotide sequence coding for this gD gene include a DNA having the nucleotide sequence shown in Fig. 2. The portion from No. 186 to No. 1016 thereof corresponds to the essential portion. Examples of the gB genes include a polypeptide having the amino acid sequence shown

10   in Fig. 3 (surface protein gB of HSV-1 strain Miyama, Japanese Patent Application No. 1-158238/1989 filed on June 22, 1989 and Japanese Patent Application No. 1-308941/1989 filed on November 30, 1989). The essential portion thereof is from Ala of No. 1 to Asp of No. 293. Examples of the DNAs containing the nucleotide sequence coding for this gB gene include a DNA having the nucleotide sequence shown in Fig.

15   4. The portion from No. 341 to No. 1219 thereof corresponds to the essential portion. The gB genes further include, for example, genes having the nucleotide sequences and the amino acid sequences deduced therefrom shown in Fig. 5 [surface protein gB of HSV-1 strain KOS, D. J. Bzik et al., Virol . 133, 301 (1984)] and Fig. 6 [surface protein gB of HSV-1 strain F, P. E. Pellet et al., J. Virol. 53, 243 (1985)]. The IL-2 genes are combined with these genes, preferably with the truncated gD and/or gB gene lacking the coding regions

20   of the transmembrane domains, whereby the fused protein genes can be constructed.

Amino acids residues in a protein may be modified by oxidation, reduction, or other dervitization without loss of activity. Furthermore, modifications of the primary structure of the protein by deletion, addition or alteration of the amino acids can be made without destroying the activity of the protein. Such modifications are included in the definition of "essential portion" as used herein so long as the bioactivity of the protein is

25   not destroyed. It is expected that such modifications may qualitatively or quantitively affect the bioactivity of the protein in the vaccines of the present invention.

IL-2 is one particularly preferred lymphokine for use in the vaccines of the present invention. Any IL-2 gene can be used as long as it codes for an IL-2 active substance. The IL-2 active substance may be any IL-2 as long as it has IL-2 activity, namely the activity of enabling the passage maintenance of T cells.

30   Examples of such substances include natural IL-2 produced in animal bodies or animal cells, recombinant IL-2 produced by recombinant technology and their related substances. In particular, human IL-2 is preferable, and more particularly, recombinant human IL-2 is preferable. When the Il-2 described above and the related substances thereof are proteins, they may have sugar chains or not.

Specifically, there may be used, for example, polypeptide (A) produced by genetic engineering

35   technique and having the amino acid sequence shown in Fig. 7 (refer to Japanese Patent Unexamined Publication No. 61-78799/1986), and a fragment having a portion of the amino acid sequence necessary for its biological or immunological activity. Examples of the fragments include a fragment lacking one amino acid residue at the amino terminus (refer to European Patent Publication No. 91539), a fragment lacking 4 amino acid residues at the amino terminal portion (refer to Japanese Patent Unexamined Publication No.

40   60-126088/1985) and a fragment lacking several amino acid residues at the carboxyl terminal portion. Further, a portion of the above polypeptide (A) may be deleted or substituted by a different amino acid(s). For example, the cystine residue at the 125-position may be replaced with a serine residue (refer to Japanese Patent Unexamined Publication No. 59-93093/1984).

The above recombinant IL-2 produced by genetic engineering technique may be a polypeptide in which

45   an Met residue is further added to the amino terminus of polypeptide (A) (refer to Japanese Patent Unexamined Publication No. 61- 78799/1986), or a mixture of polypeptide (A) and the polypeptide in which an Met residue is further added to the amino terminus of polypeptide (A) (refer to Japanese Patent Unexamined Publication No. 60-115528/1985).

The recombinant DNA (expression plasmid) containing the nucleotide sequence coding for the fused

50   protein (I) or (II) of the present invention can be prepared, for example, by the following processes.

(a) A desired truncated gene is cut out from a plasmid in which the gD or gB gene of HSV-1 strain Miyama has been cloned.

(b) A appropriate linker is added thereto as needed, followed by construction of a fused gene in which an IL-2 gene is linked to the 3′-terminal portion of the DNA.

55   (c) The resulting fused protein gene is ligated downstream from a promoter in an expression vector.

In the present invention, any vector (for example, plasmid) may be used as long as it can be replicated in an eucaryotic cell as a host. When the host is yeast, examples of such vectors include pSH19 [S. Harashima et al., Mol. Cell. Biol. 4 , 771 (1984)] and pSH19-1 (European Patent Publication No. 0235430),

and a vehicle for expression of foreign genes is obtained by inserting a promoter therein. When the host is an animal cell, the vehicle for expression of foreign genes is obtained, for example, by inserting an SV40-derived promoter, a retrovirus promoter or the like in pBR322.

As the promoter used in the present invention, any promoter is usable as long as the promoter is suitable for expression in the host used for the gene expression. When the host is yeast, it is preferred that a GLD (GAPDH) promoter, a PHO5 promoter, a PGK promoter, an ADH promoter, a PHO81 promoter and the like are used. When the host is an animal cell, an SV40-derived promoter, a retrovirus promoter and the like are preferably used.

The promoters can be prepared enzymatically from the corresponding genes. They can also be chemically synthesized.

By using the vector containing the recombinant DNA thus constructed, the eucaryotic cell is transformed.

The host includes, for example, yeast and animal cells.

Examples of the yeast include Saccharomyces cerevisiae AH22R⁻, NA87-11A and DKD-5D and Schizosaccharomyces pombe ATCC38399(h⁻ leu1·32) and TH168(h⁹⁰ ade6-M210 ura1 leu1) [M.Kishida et al., Current Genetics , 10 , 443C1986)].

Examples of the animal cells include adherent cells such as monkey cell COS-7, Vero, Chinese hamster ovary cell (CHO), mouse L cell and human FL cell, and non-adherent cells such as mouse myeloma cell (such as SP2/0), mouse YAC-1 cell, mouse MethA cell, mouse P388 cell and mouse EL-4 cell.

The transformation of the yeast is carried out according to, for example, the method described in Proc. Natl. Acad. Sci. U.S.A. , 75 , 1929 (1978). The transformation of the animal cell is carried out according to, for example, the method described in Virology, 52, 456 (1973).

The transformants (recombinants) thus obtained are cultivated by per se known methods.

When the transformants in which the host is yeast are cultivated, there is used, for example, Burkholder minimum medium [K. L. Bostian et al., Proc. Natl. Acad. Sci. U.S.A. , 77 , 4505 (1980)] as a medium. The pH of the medium is preferably adjusted to about 5 to 8. The cultivation is usually carried out at about 20 to 35°C for about 24 to 72 hours, with aeration or agitation if necessary.

When the transformants in which the host is an animal cell are cultivated, there can be used as the medium, for example, about 5 to 20% fetal calf serum-containing, MEM medium [Science , 122 , 501 (1952)], DMEM medium [Virology , 8 , 396 (1959)], RPMI1640 medium [Journal of the American Medical Association , 199 , 519 (1967)] and 199 medium [Proceeding of the Society for the Biological Medicine , 73 , 1 (1950)]. The pH is preferably about 6 to 8. The cultivation is usually carried out at about 30 to 40°C for about 15 to 60 hours, with aeration or agitation if necessary.

In the present invention, the fused proteins having both the HSV surface antigenicity and the IL-2 activity can be separated and purified by appropriate combinations of per se known separating and purifying methods. These known separating and purifying methods include methods utilizing a solubility such as salt precipitation and solvent precipitation, methods mainly utilizing a difference in molecular weight such as dialysis, ultrafitration, gel filtration and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in electric charge such as ion-exchange column chromatography, methods utilizing specific affinity such as affinity chromatography, methods utilizing a difference in hydrophobicity such as reverse-phase high performance liquid chromatography and methods utilizing a difference in isoelectric point such as isoelectric point electrophoresis.

The fused protein of an antigen other than the HSV surface protein and IL-2 can be prepared using a gene (DNA) coding for that antigen in lieu of the HSV surface protein gene, according to the methods described above.

The fused protein of the antigen used for vaccine and a lymphokine other than IL-2 can be prepared using a gene coding for the antigen and a gene coding for the lymphokine, according to the methods described above.

When the virus is a partially or completely single-stranded virus, a double-stranded DNA which is obtained by conversion with DNA polymerase can be used. When the virus is an RNA virus, there can be used a double-stranded DNA which is obtained by synthesizing a single-stranded DNA by using a reverse transcriptase and then converting the single-stranded DNA with DNA polymerase.

The host used for expression of the recombinant DNA may be a procaryotic cell such as Escherichia coli or Bacillus . However, in order to improve the immunogenicity of the antigen-lymphokine fused proteins obtained, a eucaryotic cell is advantageously used as described above.

The protein simultaneously containing the antigen used for vaccine and the lymphokine can be obtained by combining 2 kinds of proteins by chemical methods as described below, in addition to the above genetic engineering technique. Namely, for the purpose of chemically combining the antigen used for vaccine with

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the lymphokine, there can be utilized substituent groups existing in these proteins, such as amino, carboxyl, hydroxyl and sulfhydryl groups. For example, the following methods are used.

(1) A reactive amino group of one protein is condensed with a reactive carboxyl group of the other protein by dehydration in a water-soluble solvent, using a water-soluble carbodiimide reagent such as 1-ethyl-3-(3-dimethylamino- propyl)-carbodiimide or 1-cyclohexyl-3-(2-morpholinoethyl)- carbodiimide-p-toluene sulfonate.

(2) A reactive amino group of one protein is reacted with a reactive ester of N-hydroxysuccimide such as p-maleimidomethylcyclohexane-1-carboxyl-N-hydroxysuccimide ester or N-(ε-maleimidocaproyloxy)-succimide ester to maleimidate the protein, and then the resulting product is combined with a sulfhydryl group of (i) a protein obtained by reducing the other protein with dithiothreitol (DTT) or (ii) a protein obtained by introducing a sulfhydryl group in the other protein with N-succimidyl-3-(2-pyridylthio)-propionate (SPDP), to combine them through a thioether bond.

(3) Both reactive amino groups of two kinds of proteins are combined with each other by using a dialdehyde reagent such as succindialdehyde or glutaraldehyde.

(4) Sulfhydryl groups are introduced in two kinds of proteins by reduction with DTT or by SPDP, followed by reoxidation to produce a heterodimer.

Also, a desired heterodimeric protein can be efficiently produced by various combinations of these methods so that the activitoes of two kinds of proteins are not reduced.

After the completion of the combining reactions described above, the resulting hybrid proteins can be purified and separated by gel filtration chromatography using Sephadex G100 or G200, Sepharose 6B or 4B, Ultrogel AcA44 or 34, or Sephacryl S200. Further, the proteins can also be separated by a combination with affinity chromatography using an antibody column.

The antigen-lymphokine fused proteins or the antigen-lymphokine hybrid proteins obtained according to the present invention have stronger immunogenicity than the antigens not fused or combined with the lymphokines. This results from the fact that the antigen and the lymphokine simultaneously stimulate lymphocytes to promote efficiently the differentiation and proliferation of the lymphocytes, because of the presence of the antigen and the lymphokine in the same molecule. As a result, the production of antibodies to the antigens is significantly enhanced. In addition, the antigen-lymphokine proteins can also induce cell-mediated immunity. Accordingly, these proteins are particularly useful as therapeutic vaccines for virus infectious diseases observed in patients whose immunological function is lowered (for example, cancer patients and AIDS patients), and as therapeutic vaccines for prevention of recurrence diseases due to viruses inducing persistent infection (for example, herpesviruses, retroviruses and hepatitis viruses). Of course, the antigen-lymphokine proteins can also be advantageously used as preventive vaccines for prevention of infection with viruses, pathogenic protozoa and pathogenic bacteria.

The antigen-lymphokine proteins obtained according to the present invention can be (intramuscularly, subcutaneously or intracutaneously) administered in accordance with administration methods of various vaccines used for prevention of infection with viruses, pathogenic protozoa and pathogenic bacteria. In addition, these proteins can also be intravenously administered. Further, the antigen-lymphokine proteins can be used as themselves alone, as mixtures of them with conventional pharmaceutically acceptable carriers, and as liposomal preparations.

When bases, amino acids and so on are indicated by the abbreviations in this specification and the drawings, the abbreviations adopted by IUPAC-IUB Commission on Biochemical Nomenclature or commonly used in the art are employed. For example, the following abbreviations are used. When the optical isomer is capable of existing with respect to the amino acids, the L-form is represented unless otherwise specified.

DNA : Deoxyribonucleic acid

cDNA : Complementary deoxyribonucleic acid

RNA : Ribonucleic acid

mRNA : Messenger RNA

A : Adenine

T : Thymine

G : Guanine

C : Cytosine

dATP : Deoxyadenosine triphosphate

dTTP : Deoxythymidine triphosphate

dGTP : Deoxyguanosine triphosphate

dCTP : Deoxycytidine triphosphate

ATP : Adenosine triphosphate

EDTA : Ethylenediaminetetraacetic acid
SDS : Sodium dodecyl sulfate
DTT : Dithiothreitol
Gly : Glycine (G)
5   Ala : Alanine (A)
Val : Valine (V)
Leu : Leucine (L)
Ile : Isoleucine (I)
Ser : Serine (S)
10 Thr : Threonine (T)
Cys : Cysteine (C)
1/2 Cys: Half cysteine
Met : Methionine (M)
Glu : Glutamic acid (E)
15 Asp : Aspartic acid (D)
Lys : Lysine (K)
Arg : Arginine (R)
His : Histidine (H)
Phe : Phenylalanine (F)
20 Tyr : Tyrosine (Y)
Trp : Tryptophan (W)
Pro : Proline (P)
Asn : Asparagine (N)
Gln : Glutamine (Q)
25 Ap^r : Ampicillin-resistant gene
Tc^r : Tetracycline-resistant gene
ARS 1: Autonomous replication sequence 1

With respect to the proteins of the present invention, a portion of the amino acid sequence may be modified, namely there may be addition, elimination or substitution by a different amino acid(s) as long as
30 the immunogenicity is not lost.

The present invention will hereinafter be described in detail with the following Reference Examples and Examples. It is understood of course that these Reference Examples and Examples are merely illustrative and are not intended to limit the scope of the invention.

Transformant CHO-HDL-1-5 obtained in Example 3 described below and bearing plasmid pHDLdhfr1
35 was deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan (FRI) under the accession number FERM BP-2506 on July 7, 1989. This microorganism was also deposited with the Institute for Fermentation, Osaka, Japan (IFO) under the accession number IFO 50192 on June 26, 1989.

Transformant Escherichia coli DH1/pHSD BJ-1 bearing plasmid PHSD BJ-1 described in Reference
40 Example mentioned below was deposited with the FRI under the accession number FERM BP-1784 on March 9, 1988. This microorganism was also deposited with the IFO under the accession number IFO 14730 on February 23, 1988.

Transformant Saccharomyces cerevisiae NA74-3A($\bar{p}$)/pGFE213 bearing plasmid pGFE213 described in Example 1 mentioned below was deposited with the FRI under the accession number FERM BP-2095 on
45 October 11, 1988. This microorganism was also deposited with the IFO under the accession number IFO 10460 on September 19, 1988.

Animal cell SP-neo-HSD-39 described in Example 6 mentioned below was deposited with the FRI under the accession number FERM BP-2809 on March 16, 1990. This microorganism was also deposited with the IFO under the accession number IFO 50231 on March 1, 1990.

50 Animal cell SP-neo-HDL-245 described in Example 8 mentioned below was deposited with the FRI under the accession number FERM BP-2810 on March 16, 1990. This microorganism was also deposited with the IFO under the accession number IFO 50232 on March 1, 1990.

Transformant Escherichia coli K12 DH1/pTB652 bearing plasmid pTB652 described in Example 5 mentioned below was deposited with the FRI under the accession number FERM BP-1373 on September 5,
55 1986. This microorganism was also deposited with the IFO under the accession number IFO 14539 on August 29, 1986.

Transformant Escherichia coli JM109/pVGL4 bearing plasmid pVGL4 described in Example 15 mentioned below was deposited with the FRI under the accession number FERM BP-2977 on June 20, 1990.

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This microorganism was also deposited with the IFO under the accession number IFO 15049 on June 13, 1990.

Reference Example 1

Preparation of Plasmid pHSG396SgD

A DNA coding for the 20 amino acid residues from the N-terminus of gD, namely the 73-bp DNA fragment shown in Fig. 8 was chemically synthesized, and inserted into vector pUC8 digested with BamHI and.HindIII.

The resulting pUC8 BamHI-HindIII73 was digested with BamHI and NcoI to obtain a 73-bp fragment. On the other hand, a NcoI-SacI DNA fragment of about 1.28 kb was obtained from cloning plasmid pUC18gD having an HindIII-NruI fragment [plasmid pHSD BJ-1 (IFO 14730, FERM BP-1784 origin] of about 1.4 kb containing the gD-coding region of HSV. The above 73-bp fragment and the above NcoI-SacI DNA fragment were reacted with a BamHI-SacI digest of plasmid vector pHSG396 (Takara Shuzo) to prepare subcloning plasmid pHSG396SgD.

Reference Example 2

(1) Preparation of Virus DNA of Varicella-zoster Virus, Kuzuhara Strain

Flow 2000 cells (of human fetal lung origin) which were infected with varicella-zoster virus, Kuzuhara strain (VZV, KY strain) were inoculated at 10:1 to a monolayer (1575 cm^2) of Flow 2000 cells, followed by incubation in GIT medium (Nihon Pharmaceutical) at 37°C. When at least 50% of the cells showed cytopathic effect, the cells were treated with trypsin-EDTA, and the infected cells were recovered, followed by centrifugation at low speed (1,500 rpm, 10 minutes) to remove a supernatant. To pellets of the resulting infected cells was added 0.3 ml of PBS (0.8% NaCl, 0.02% KCl, 0.115% Na$_2$HPO$_4$, 0.02% KH$_2$PO$_4$, pH 7.2) to obtain 0.66 ml of a suspension.

To the suspension was added 0.66 ml of low melting point agarose [1% low melting point agarose (FMC), 10 mM Tris HCl (pH 8.0), 1 mM EDTA), and the mixture was poured into a template (57 mm X 2 mm X 9 mm) to obtain an agarose block containing the infected cells. The agarose block was incubated in 15 ml of lysis buffer [1% SDS, 100 mM EDTA, 20 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mg/ml Proteinase K] at 37°C overnight. The agarose block was transferred into a buffer which was prepared by removing SDS and Proteinase K from the above lysis buffer, and incubated overnight again. Then, the culture was allowed to stand in TE buffer (50 mM Tris-HCl, 500 mM EDTA, pH 8.0) at 4°C until it was subjected to electrophoresis.

The above agarose block containing virus DNA was embedded in a 1% agarose gel [1% GTG agarose (FMC), 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA (pH 8.0)], and electrophoresis was carried out by using a pulsed field gel electgrophoresis apparatus (LKB) at 240 V at a pulse of 60 sec for 18 hours.

After electrophoresis, the gel was stained in 0.5 μg/ml ethidium bromide solution, and the virus DNA which appeared near 120 kb was cut out together with the agarose gel. The agarose gel was immersed in 30 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and allowed to stand at 4°C for 2 hours. Then, the TE buffer was exchanged for a fresh one. After standing for 2 hours, the buffer was exchanged for a fresh one once more, followed by standing overnight. The agarose gel was washed with TE buffer once, and then immersed in 30 ml of a restriction enzyme reaction solution [10 mM Tris-HCl (pH 7.5), 7 mM MgCl$_2$, 100 mM NaCl, 7 mM 2-ME(mercaptoethanol), 0.01%BSA (bovine serum albumin)], followed by standing at 4°C for 2 hours. After this reaction solution was exchanged for a fresh one (10 ml), 1,200 units of restriction enzyme HindIII (Takara Shuzo) was added thereto, followed by standing at 37°C for 5 hours.

After the reaction, the HindIII-digested virus DNA was electrically eluted from the agarose gel in a dialysis tube. About 2 ml of the resulting eluate was concentrated to 200 μl by a Centricon (Amicon), and ethanol was added thereto to precipitate the DNA. The precipitate was dissolved in 20 μl of restriction enzyme buffer (the same as described above in composition), and 10 units of XbaI and 10 units of HindIII (Takara Skhuzo) were added thereto, followed by reaction at 37°C for 2 hours. The resulting reaction solution was subjected to electrophoresis in a 0.7% GTG agarose gel (FMC) as it is. As a result, there were

detected fragments having a size similar to that reported by Davison et al. [J. Gen. Virol. 67 , 1759 (1986)].

(2) Preparation of Plasmid Containing DNA Fragment of VZV, KY Strain

Of the XbaI-HindIII-digested fragments of the DNA of the VZV, KY strain, which were obtained in (1), fractions of about 8 to 10 kb were cut out of the agarose gel and electrically eluted, followed by phenol treatment and ethanol precipitation. About 50 ng of the DNA fragments were mixed with about 30 ng of pUC18 cleaved with XbaI and HindIII, and the mixture was incubated in 25 μl of a reaction solution [66 mM Tris-HCl, pH 7.6, 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 20 units of T4 DNA ligase (Takara Shuzo)] at 16°C overnight. Then, using the resulting solution, Escherichia coli JM109 was transformed. Plasmids contained in a white colony which appeared on an agar plate containing 100 μg/ml ampicillin, 0.2% X-gal and 10 mM IPTG were isolated by the alkali extraction method (T. Maniatis et al., Molecular Cloning , Cold Spring Harbor Laboratory, U.S.A., 1982), and the size of the XbaI-HindIII-digested fragments of cloned VZV DNA was examined by electrophoresis using a 0.7% agarose gel. A clone (pVHX7) into which a fragment of about 8.5 kb was inserted was selected, and the restriction map of the fragment was prepared. As a result, the map was similar to that reported by Davison et al., and it was anticipated that the fragment would contain a glycoprotein gpI gene (Fig. 16-1).

A 5.2 kb fragment obtained from the XbaI-SmaI digests of the above fragment was subcloned into the XbaI/SmaI site of pUC18 to prepare pCU18gpI (Fig. 16-1).

With respect to insert of pUC18gpI, the nuclectide sequence of the region of about 2.1 kb from the smaI site was determined by the dideoxynucleotide synthetic chain termination method. The results showed that a VZVgpI protein was coded in the above region (Fig. 17).

An amino acid sequence deduced from the above nucleotide sequence is shown in Fig. 17. The nucleotide sequence of the above region was very similar to that reported by Davison et al. However, there were observed mutations in four bases [T of No. 196 (this invention) → C (Davison); C of No. 276 → T; T of No. 1969 → C; and T of No. 2040 → lacking] (mutation in one amino acid: the 40-position is Thr in the report of Davison, but Ile in this invention).

(3) Construction (I) of Plasmid for Expression of VZVgpI Gene: Construction of Truncated gpI Transient Expression Plasmid

(i) pUC18gpI (Fig. 16-1) was digested with AvaI and NcoI to isolate a 0.35-kb fragment from -53 to +293 of a translation initiating codon of gpI. pUC19Nco which was obtained by inserting an NcoI linker (Pharmacia) into the smaI site of vector pUC19 was cleaved with NcoI and BamHI. The resulting vector was ligated to the above 0.35-kb NcoI-AvaI fragment with T4 DNA ligase once, followed by reaction with BamHI, AvaI and T4 DNA ligase in order. Finally, ring closure was conducted with T4 DNA ligase to prepare pUC19gpINco (Fig. 16-2).

pUC19gpINco was reacted with xbaI, Klenow fragment E. coli DNA polymerase I (Klenow polymerase) and KpnI in order to open the ring. Thus, a 0.35-kb fragment was obtained. On the other hand, pUC18Nhe which was prepared by inserting an Nhe linker into pUC18 was reacted with EcoRI, Klenow DNA polymerase and KpnI in order to obtain a ring-opened vector. The resulting vector was ligated to the above 0.35-kb fragment with T4 DNA ligase to prepare pUC18NhegpINco (Fig. 16-2).

(ii) pUC18gpI was digested with smaI and NcoI to obtain a 1.8-kb fragment, and pUC18NhegpINco was reacted with NheI, Klenow DNA polymerase and NcoI in order to obtain a 3.1-kb vector. The above 1.8-kb fragment was ligated to the above 3.1-kb vector with T4 DNA ligase to obtain plasmid pUC18gpISma (Fig. 16-3).

The plasmid PUC18gPISma was cleaved with EcoT22I and the termini of the cleaved fragment were changed to flush ends, followed by insertion of an NheI linker to obtain plasmid pUC18NhegpIEcT (Fig. 16-3).

(iii) The plasmid pUC18NhegpIEcT was digested with XbaI to obtain a 2.1-kb fragment, and this fragment was treated with Klenow DNA polymerase. On the other hand, pTB701 [a vector which was obtained by removing a c kinase gene from pTB652, Ono et al., Science 236 , 1116-1120 (1987)] was cleaved with EcoRI, followed by treatment with Klenow DNA polymerase to obtain a vector. The above fragment treated with Klenow DNA polymerase was ligated to the resulting vector with T4 DNA ligase to prepare expression plasmid pTBgpIEcT (Fig. 16-4).

(iv) pUC18gpI was cleaved with SmaI and SacI, and the portion of about 0.45 kb on the 3'-terminal side

of the gpl gene was digested with exonuclease III. Then, the resulting fragment was treated with mung bean nuclease and Klenow DNA polymerase to change the termini thereof to flush ends, followed by ring closure with T4 DNA ligase to prepare pUC18SS60 (Fig. 16-5).

pUC18SS60 was cleaved with KpnI and partially digested with EcoRI to obtain a 2.3-kb fragment. The termini of this fragment were changed to flush ends with T4 DNA polymerase, and an NheI linker (New England Biolabs) was ligated thereto, followed by trimming with NcoI and NheI to prepare a 1.3-kb fragment. The resulting fragment was ligated to a vector which was obtained by cleaving pUC18NhegplEcT with NcoI and NheI to prepare pUC18gplSS60 (Fig. 16-5).

(v) pUC18gplSS60 was partially digested with EcoRI, and DNA fragments each of which was cleaved only at one portion were recovered. Then, the fragments were treated with Klenow DNA polymerase, followed by ring closure with T4 DNA ligase. From these was selected clone pUC18SS60-E7 in which the EcoRI site derived from pUC18 in pUC18SS60 disappeared (Fig. 16-6).

The termini of a 2.7-kb fragment obtained by treating PUC18SS60-E7 1with XbaI were changed to flush ends with Klenow DNA polymerase. On the other hand, pTB701 was cleaved with EcoRI and then the termini of the fragment were changed to flush ends with Klenow DNA polymerase to obtain a vector. The above fragment was ligated to the resulting vector to prepare expression plasmid pTBgplE7-17 (Fig. 16-6).

(4) Construction (II) of Plasmid for Expression of VZVgpl Gene : Construction of Truncated gpl Stable Expression Plasmid

Expression plasmid pTB564 of a hamster dihydrofolate reductase (hDHFR) was digested with ClaI to obtain a 1.9-kb fragment. The termini of the resulting fragment were changed to flush ends with Klenow DNA ligase. The expression plasmid pTB564 was prepared by ligating a 0.9-kb fragment, a 2.4-kb fragment and a 0.8-kb fragment to one another with T4 DNA ligase, which were obtained by digesting pTB348, pTB399 and pTB401 [R. Sasada et al., Cell Structure and Function 12 , 205 (1987)] with PstI and BamHI, SalI and BamHI, and SalI and PstI, respectively. On the other hand, pTBgPIE7-17 was cleaved with SalI, and then the termini of the fragment were changed to flush ends with Klenow DNA polymerase to obtain a vector. The above fragment was ligated to the resulting vector to prepare expression plasmid pTBE7dhfr4 (Fig. 16-7).

Example 1

Construction of HSV-1 Truncated gD Gene

The plasmid vector pHSG396SgD (Reference Example) having the HSV-1 strain Miyama gD gene was digested with restriction enzymes XhoI and XbaI to obtain a DNA fragment of about 1.35 kb, followed by further digestion with restriction enzyme HinfI to obtain an XhoI-HinfI fragment of about 0.91 kb. A 12-bp DNA fragment shown in Fig. 9 containing a stop codon was chemically synthesized, and reacted with the above XhoI-HinfI fragment and an XhoI-SacI digest of plasmid vector pHSG397 (Takara Shuzo) to prepare subcloning plasmid pHSG397SgDΔHinf. The resulting plasmid was digested with restriction enzymes XhoI and SacI to obtain an XhoI-SacI DNA fragment of about 0.92 kb. The fragment thus obtained was reacted with an XhoI-SacI digest of the plasmid PGFE213 (IFO 10460, FERM BP-2095 origin) described in Japanese Patent Application No. 63-180114/1988 and Reference Example 1 of Japanese Patent Application No. 63-317546/1988 to obtain expression plasmid pHSD104ΔHinf (refer to Fig. 9).

Example 2

Construction of Gene Expression Plasmid for Fused Protein Composed of HSV-1 Truncated gD and I1-2

The subcloning plasmid pHSG397SgDΔHinf constructed in Example 1 was digested with XhoI, and a Klenow fragment was allowed to react on the digest, followed by insertion of an EcoRI linker (pGGAATTcc) (NEB) to obtain pHSG397SgDΔHinfE. The resulting plasmid was digested with HinfI to obtain a DNA fragment of about 0.95 kb, on which a Klenow fragment is allowed to react, followed by addition of an NheI

linker (pCGCTAGCG) (Pharmacia) using T4 DNA ligase (Takara Shuzo). The resulting fragment was further digested with EcoRI and NheI to obtain an EcoRI-NheI fragment of about 0.9 kb coding for truncated gD lacking 94 amino acid residues from the C-terminus.

Then, animal cell expression plasmid pTB399 [Japanese Patent Unexamined Publication No. 61-63282/1986, R. Sasada et al., Cell Structure and Function 12 , 205 (1987)] of human interleukin 2 awas digested with EcoRI and HindIII to obtain a fragment, which was further digested with HgiAI to obtain a fragment of about 0.45 kb. T4 DNA polymerase was allowed to react on the fragment thus obtained, followed by addition of the above NheI linker. The resulting fragment was further digested with BamHI and NheI to obtain an NheI-BamHI fragment of about 0.43 kb containing the coding region of mature human interleukin 2.

The two fragments described above were reacted with a fragment of about 3.9 kb obtained by EcoRI-BglII digestion of pTB399 to obtain an expression plasmid pHDL201.

Further, in order to express the above fused protein in CHO cells and to enable gene amplification, a DNA fragment containing a fused gene of IL-2 and truncated gD of about 2.9 kb which was obtained by digesting the plasmid pHDL201 with ClaI was inserted into the ClaI site of dihydrofolate reductase (DHFR) gene expression plasmid pTB348 (refer to Japanese Patent Unexamined Publication No. 61-63282/1986) to obtain plasmid pHDLdhfrI (refer to Fig. 10).

The nucleotide sequence of the resulting fused gene is shown in Fig. 11, and the amino acid sequence deduced therefrom is shown in Fig. 12.

Example 3

Gene Expression of Fused Protein Composed of HSV-1 Truncated gD and IL-2 in Animal Cell

Using the plasmid pHDLdhfrI constructed in Example 2, CHO cell DHFR$^-$ strain [G. Urlaub and L. A. Chasim, Proc. Natl. Acad. Sci. U.S.A. 77 , 4216-4220 (1980)] was transformed by the calcium phosphate method [C. M. Gorman et al, Science 221 551-553 (1983)] to obtain a transformant which was converted to DHFR$^-$.

The resulting transformant CHO-HDL-1-5 (IFO 50192, FERM BP-2506) was cultivated in Dulbecco MEM medium (Gibco) containing 10% fetal calf serum (Whittaker M. A. Bioproducts) so as to become confluent. Then, the medium was exchanged for a methionine-free medium, and 25 μCi/ml of ^{35}S-methionine was added thereto, followed by cultivation overnight.

After a supernatant of the culture was recovered, 5 μl/ml of supernatant of rabbit anti-HSV-1 (MacIntyre) serum (Dakopatt) or 10 μl/ml of supernatant of rabbit anti-human IL-2 serum was added to the supernatant, followed by cultivation at 4°C for 2 hours. Then, protein A-Sepharose (Pharmacia) was added thereto, and cultivation was further carried out at 4°C for 2 hours, followed by centrifugation to recover a precipitate. The precipitate was washed with a buffer containing 0.05% NP-40, and Laemmli buffer was added thereto, followed by heating at 100°C for 5 minutes. After cooling, a supernatant was recovered by centrifugation and subjected to SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel was dried, and subjected to autoradiography. As a result, it was revealed that a product of about 45 to 60 k daltons which was reactive to both anti-HSV-1 and anti-IL-2 antibodies was produced.

Example 4

Detection of IL-2 Activity in Expressed Product of Gene Coding for Fused Protein Composed of HSV-1 Truncated gD and IL-2

With respect to the culture of the transformant in which the expression of the fused protein composed of truncated gD and human IL-2 was observed in Example 3, the IL-2 activity was measured by the modified MTT method [H. Tada et al., J. Immunol. Methods 93 , 157 (1986)], using IL-2-dependent cell strain NKC3.

As a result, the IL-2 activity was only detected in the culture supernatant of the cell in which the fused gene was introduced.

Example 5

Construction of Plasmid for Expression of HSV-1 Truncated gD Gene in Myeloma Cell

The plasmid pHSG397SgDΔHinfE constructed in Example 2 was digested with restriction enzyme EcoRI to obtain a fragment of about 0.9 kb coding for truncated gD. The fragment thus obtained was inserted into the EcoRI site of pTB701 [a vector obtained by removing a C-kinase gene from plasmid pTB652 described in Ono et al., Science 236 , 1116-1120 (1987)], thereby obtaining a truncated gD expression plasmid pHSD207 having a long terminal report and the early promoter of SV40.

Then, plasmid pMAMneo (Clontec) having a neomycin-resistant gene was digested with BamHI to obtain a fragment of about 2.8 kb containing the early promoter of SV40, the neomycin-resistant gene and a polyadenylation site. This fragment was subcloned to the BamHI site of pHSG396 (Takara Shuzo), followed by further digestion with restriction enzymes ClaI and SalI to obtain a ClaI-SalI fragment of about 2.8 kb containing the neomycin-resistant gene. The resulting fragment was reacted with a ClaI-SalI digest (about 5.1 kb) of the above plasmid pHSD207 to obtain an expression plasmid pHSDneol of about 7.9 kb (refer to Fig. 13).

Example 6

Expression of HSV-1 Truncated gD Gene in Myeloma Cell

Using the plasmid pHSDneol constructed in Example 5, mouse myeloma cell Sp2/0-Ag14 (Dainippon Pharmaceutical) was transformed by electroporation using a Gene Pulser (Bio-Rad), followed by cultivation in RPMI1640 medium (Gibco) containing 400 μg/ml of G418 (Gibco) and 10% fetal calf serum to obtain G418-resistant transformants. A culture supernatant of the transformants was screened according to an enzyme immunoassay by a sandwich method using a microplate (Nunc) coated with rabbit anti-HSV-1 serum (Dakopatt) and biotinyl anti-HSV-1 & -2 antibody (Chemicon) to obtain clones in which truncated gD was expressed.

The resulting high expression clone SP-neo-HSD-39 was cultivated in RPMI1640 medium (Gibco) containing 10% fetal calf serum (Whittaker M. A. Bioproducts), and then the medium was exchanged for a methionine-free medium, and 25 μCi/ml of ^{35}S-methionine was added thereto, followed by cultivation overnight.

After a supernatant of the culture was recovered, 5 μl/ml of supernatant of rabbit anti-HSV-1 serum (Dakopatt) was added to the supernatant, and the mixture was incubated at 4°C for 2 hours, followed by centrifugation to recover a precipitate. The precipitate was washed with a buffer containing 0.05% NP-40, and Laemmli buffer was added thereto, followed by heating at 100°C for 5 minutes. After cooling, a supernatant was recovered by centrifugation and subjected to SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel was dried, and subjected to autoradiography. As a result, it was revealed that a product of about 40 to 50 k daltons which was reactive to an anti-HSV-1 antibody was produced.

Example 7

Construction of Gene Expression Plasmid Fused Protein Composed of HSV-1 Truncated gD and IL-2 in Myeloma Cell

The plasmid pHDL201 constructed in Example 2 was digested with restriction enzymes SalI and EcoRI to obtain a fragment of about 3.9 kb containing a fused gene composed of truncated gD and IL-2. On the other hand, the truncated gD expression plasmid pHSDneol having the neomycin-resistant gene in Example 5 was digested with SalI and EcoRI to obtain a fragment of about 4.4 kb containing the neomycin-resistant gene. These two fragments were reacted with each other to obtain expression plasmid pHDLneol of the truncated gD-IL-2 fused gene having the neomycin-resistant gene (refer to Fig. 14).

Example 8

Gene Expression of Fused Protein Composed of HSV-1 Truncated gD and IL-2 in Myeloma Cell

Using the plasmid pHDLneol constructed in Example 7, mouse myeloma cell Sp2/0-Ag14 (Dainippon Pharmaceutical) was transformed by electroporation using a Gene Pulser (Bio-Rad), followed by cultivation in RPMI1640 medium (Gibco) containing 200 µg/ml of G418 (Gibco) and 10% fetal calf serum to obtain G418-resistant transformants. A culture supernatant of the transformants was screened according to an enzyme immunoassay by a sandwich method using a microplate (Nunc) coated with rabbit anti-HSV-1 serum (Dakopatt) and biotinyl anti-HSV-1 & -2 antibody (Chemicon) to obtain clones in which truncated gD was expressed.

Of the clones, Sp-neo-HDL-245 relatively high in expression amount was cultivated in serum-free medium ASF104 (Ajinomoto), and 1 ml of a supernatant thereof was concentrated by Ultrafree PF (Millipore). Then, Laemmli buffer was added thereto to 50 µl, followed by heating at 100°C for 5 minutes. After cooling, SDS-polyacrylamide gel electrophoresis was conducted, and further the western blotting method was carried out using rabbit anti-HSV-1 serum (Dakopatt) and rabbit anti-human IL-2 serum (Genzyme). As a result, a band recognized by all antibodies was specifically detected.

Example 9

Expression of HSV-1 Trucated gD Gene in Animal Cell

Expression plasmid pHSDdhfr1 of HSV-1 truncated gD gene for animal cells was prepared as described in Reference Examples 1 and 2 and Example 1 of Japanese Patent Application No. 1-233728/1989, and transformant CHO-HSD-1-7 was obtained as described in Example 2 of the same application. The details thereof will hereinafter be described.

The plasmid pHSG397SgDΔHinf shown in Example 1 was digested with XhoI and SacI, and then T4 DNA polymerase was allowed to react on the digest to obtain a fragment of about 0.9 kb containing the truncated gD gene, both ends of the fragment being flush.

Then, plasmid pTB399 [Japanese Patent Unexamined Publication No. 61-63282/1986; R. Sasada et al., Cell Structure and Function 12, 205 (1987)] was digested with restriction enzymes EcoRI and BglII, and then T4 DNA polymerase was allowed to react on the digest to obtain a fragment of about 3.9 kb both ends of which are flush. The resulting fragment was reacted with the above fragment containing truncated gD in the presence of T4 DNA ligase to obtain expression plasmid pHSD2O9.

Then, in order to express the gene in CHO cells and to enable gene amplification, a fragment of about 2.4 kb which was obtained by digesting the plasmid pHSD2O9 with restriction enzyme ClaI was inserted into the ClaI site of plasmid pTB348 (refer to Japanese Patent Unexamined Publication No. 61-63282/1986) to obtain plasmids pHSDdhfr1 and pHSDdhfr2.

Using the plasmid pHSDdhfr1, CHO cell DHFR⁻ strain [G. Urlaub and L. A. Chasim. Proc. Natl. Acad. Sci. U.S.A. 77, 4216-4220 (1980)] was transformed by the calcium phosphate method [C. M. Gorman et al, Science 221, 551-553 (1983)] to obtain a transformant which was converted to DHFR⁻.

Example 10

Purification of HSV-1 Truncated gD (t-gD)

The transformant CHO-HSD-1-7 obtained in Example 9 was cultivated in serum-free medium ASF104 (Ajinomoto) so as to give a confluent state. Then, 5 l of the culture supernatant was dialyzed against 20 mM Tris-HCl (pH 8.0) buffer, followed by addition of ammonium sulfate to obtain a 20% saturated concentration. The resulting solution was subjected to a Butyl-Toyopearl column (100 ml in bed capacity, Ø 2.6 X 19 cm) equilibrated with 20% saturated ammonium sulfate/20 mM Tris-HCl (pH 8.0) buffer, and then the column was washed with the same buffer. Subsequently, t-gD was eluted by a concentration gradient (totaled 800 ml) from 20% to 0% ammonium sulfate. t-gD fractions (70 ml) eluted at saturated ammonium

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sulfate concentrations of about 3 to 5% were concentrated to 4 ml with an ultrafiltration membrane (DIAFLO; Amicon). The resulting solution was subjected to a Sephacryl S-300 column (198 ml in bed capacity, ϕ 1.6 X 98.5 cm) equilibrated with PBS, and t-gD fractions were collected as a purified sample (3.5 mg/16 ml).

Example 11

Purification of Fused Protein (t-gD-IL-2) Composed of HSV-1 Truncated gD and IL-2

The transformant CHO-HDL-1-5 obtained in Example 3 was cultivated in serum-free medium ASF104 (Ajinomoto) so as to give a confluent state. Then, 5 l of the culture supernatant was dialyzed against 20 mM Tris-HCl (pH 8.0) buffer, followed by addition of ammonium sulfate to obtain a 20% saturated concentration. The resulting solution was subjected to a Butyl-Toyopearl 650 column (100 ml in bed capacity, ϕ 2.6 X 19cm) equilibrated with 20% saturated ammonium sulfate/20 mM Tris-HCl (pH 8.0) buffer, and then the column was washed with the same buffer. Subsequently, t-gD-IL-2 was eluted by a concentration gradient (totaled 800 ml) from 20% to 0% ammonium sulfate. t-gD fractions (70 ml) eluted at saturated ammonium sulfate concentrations of about 0% were concentrated to 4 ml with an ultrafiltration membrane (Amicon). The resulting solution was subjected to a Sephacryl S-300 column (198 ml in bed capacity, ϕ 1.6 X 98.5 cm) equilibrated with PBS, and t-gD-IL-2 fractions were collected as a purified sample (2.8 mg/14 ml).

Example 12

Immunogenicity of Fused Protein (t-gD-IL-2) Composed of HSV-1 Truncated gD and IL-2

(1) Determination of Anti-HSV Antibodies

Each of truncated gD (t-gD) obtained in Example 10 and t-gD-IL-2 obtained in Example 11, alone or adsorbed on alum adjuvant (final concentration 0.5 mg/ml, pH 7.0), was abdominally subcutaneously administered in an amount of 0.2 ml/mouse to BALB/c mice (female, 6 weeks old, Charles River). After 5 weeks, blood was collected and serum samples were prepared. The anti-HSV antibodies were determined by the following method.

An inactivated HSV-coated microplate of a human anti-HSV antibody determination kit (Herpes Stat, Whittaker Bioproducts, Lot No. 002706) was blocked with PBS containing 20% FCS at room temperature for 2 hours, followed by washing 3 times with PBS containing 0.05% Tween 20 (PBS-Tween). To this plate was added 100 μl/well of the serum sample diluted with 20% FCS/40 mM Tris-HCl (pH 7.5)/5% NaCl/0.05% Tween 20, followed by incubation at room temperature for 1 hour. The plate was washed 6 times with PBS-Tween, and then 100 μl of a 1,000-fold dilution of a peroxidase-labeled anti-mouse IgG antibody (HPR-conjugated rabbit X mouse IgG [H + L], Zymed Laboratories, Lot No. 80801651) was added to each well, followed by incubation at room temperature for 30 minutes. The plate was washed 6 times with PBS-Tween, and then 100 μl of a substrate solution [2 mg/ml o-phenylenediamine/0.02% H_2O_2/0.1 M citrate buffer (pH 4.5)] was added to each well, followed by reaction for 10 minutes. After 200 μl of 2N sulfuric acid was added to each well to terminate color development, the absorbance was measured at 492 nm. (2) Comparison of Antibody Productivity of t-gD with That of t-gD-IL-2.

The titer of the anti-HSV antibody in the serum sample was calculated using mouse anti-gD monoclonal antibody M42 [Koji Inoue, Osaka University Medical Magazine 36 (No.4), 69 (1987)] as a standard antibody in the following manner. The antibody titer of the M42 antibody (1.9 mg/ml) was arbitrarily defined as to 1900 mU/ml, and the titer of the anti-HSV antibody was determined from the ratio of the dilution of M42 giving the 50% value (about 1) of the maximum absorbance ($\geq$ 2.0) given by the 4-fold dilution of M42 to that of the serum sample. Mean values for groups each consisting of 10 mice are shown in Table 1.

Table 1

		Antibody titer (mU/ml)	
Antigen	Dose (μg)	Alum (-)	Alum (+)
t-gD	0.35	-	76
	1.7	<5	228
t-gD-IL-2	1.0	-	513
	5.0	285	1,653
Control	-	-	<5

As apparent from Table 1, when the antigen was administered alone [Alum (-)], t-gD could hardly induce the antibody. However, t-gD-IL-2 significantly exhibited the antibody productivity. These results revealed that IL-2 combined with t-gD achieved a strong adjuvant activity. When the Alum adjuvant was used [Alum (+)], it was observed that t-gD produced the antibody (228 mU/ml on administration of 1.7 μg). However, the high antibody titer was obtained by t-gD-IL-2 (513 mU/ml on administration of 1.0 μg), and the effect of IL-2 addition was observed.

Example 13

Immunogenicity of Fused Protein (t-gD-IL-2) Composed of HSV-1 Truncated gD and IL-2

(1) Determination of Anti-HSV Antibodies

Each of truncated gD (t-gD) obtained in Example 10 and t-gD-IL-2 obtained in Example 11, alone, mixed with equimolar human recombinant IL-2 (rIL-2; 1.21 mg/ml, Takeda Chemical Industries, Lot No. H-609-035) or adsorbed on alum adjuvant (final concentration 0.5 mg/ml, pH 7.0), was abdominally sub-cutaneously administered in an amount of 0.2 ml/mouse to BALB/c mice (female, 8 weeks old, Charles River). After 5 weeks, blood was collected to prepare serum samples. When immunization was carried out twice, the antigen was administered again 4 weeks after the first administration, and blood was collected 2 weeks after the second administration. The anti-HSV antibodies were determined by the following method.

An inactivated HSV-coated microplate of a human anti-HSV antibody determination kit (Herpes State, Whittaker Bioproducts, Lot No. 002706) was blocked with PBS containing 20% FCS at room temperature for 2 hours, followed by washing 3 times with PBS containing 0.05% Tween 20 (PBS-Tween). To this plate was added 100 μl/well of the serum sample diluted with 20% FCS/40 mM Tris-HCl (pH 7.5)/5% NaCl/0.05% Tween 20, followed by incubation at room temperature for 1 hour. The plate was washed 6 times with PBS-Tween, and then 100 μl of a 1,000-fold dilution of a peroxidase-labeled anti-mouse IgG antibody (HPR-conjugated rabbit X mouse IgG [H + L], Zymed Laboratories, Lot No. 80801651) was added to each well, followed by incubation at room temperature for 30 minutes. The plate was washed 6 times with PBS-Tween, and then 100 μl of a substrate solution [2 mg/ml o-phenylenediamine/0.02% H_2O_2/0.1 M citrate buffer (pH 4.5)] was added to each well, followed by reaction for 10 minutes. After 200 μl of 2N sulfuric acid was added to each well to terminate color development, the absorbance was measured at 492 nm.

(2) Comparison of Antibody Productivity of t-gD with That of t-gD-IL-2

The titer of the anti-HSV antibody in the serum sample was calculated using mouse anti-gD monoclonal antibody M42 [Koji Inoue, Osaka University Medical Magazine 36 (No.4), 69 (1987)] as a standard antibody in the following manner. The antibody titer of the M42 antibody (1.9 mg/ml) was arbitrarily defined as to

1900 mU/ml, and the titer of the anti-HSV antibody was determined from the ratio of the dilution of M42 giving the 50% value (about 1) of the maximum absorbance ($\geq$ 2.0) given by the 8-fold dilution of M42 to that of the serum sample. Mean values for groups each consisting of 10 mice are shown in Table 2. The range represented by $\pm$ shows a standard deviation.

Table 2

Antigen (Dose)	Antibody titer (mU/ml)
Control [PBS]	7
t-gD (1 μg)	<15
t-gD (5 μg)	9 ± 4
t-gD (1 μg) X 2	1,018 ± 1,833
t-gD (1 μg) + IL-2 (0.25 μg)	23 ± 29
t-gD (5 μg) + IL-2 (1.25 μg)	35 ± 38
t-gD-IL-2 (1 μg)	400 ± 292
t-gD-IL-2 (5 μg)	692 ± 442
t-gD-IL-2 (1 μg) X 2	46,183 ± 38,443
t-gD (1 μg)-Alum (125 μg)	341 ± 267
t-gD (5 μg)-Alum (125 μg)	481 ± 451

As apparent from Table 2, when the antigen was once administered alone (Alum-) t-gD could hardly induce the antibody. However, t-gD-IL-2 significantly exhibited the antibody productivity, even when it was administered once. When the mixtures of t-gD and equimolar rIL-2 were administered once, the slight antibody production was only observed. These results revealed that IL-2 combined with t-gD achieved a strong adjuvant activity. When the Alum adjuvant was used (t-gD-Alum), it was observed that t-gD produced the antibody (341 mU/ml on administration of 1 μg and 481 mU/ml on administration of 5 μg). Compared to the antibody titers (400 mU/ml on administration of 1 μg and 692 mU/ml on administration of 5 μg) given by t-gD-IL-2, it was shown that the adjuvant effect due to IL-2 addition was not less than that of alum (125 μg/mouse).

(3) Determination of Killer Activity.

The killer activity was determined by the ^{51}Cr releasing method. The preparation of effector cells and the labeling of target cells with ^{51}Cr were performed according to the methods described in S. Hinuma et al., Immunology 159 , 251 (1986). Each of t-gD (5 μg), the mixture of t-gD (5 μg) and recombinant human IL-2 (rIL-2) (1.25 μg), and t-gD-IL-2 (5 μg) was dissolved in 200 μl of PBS, and the resulting solutions were abdominally subcutaneously administered to BALB/c mice (4 mice per group). After 5 weeks, spleens were obtained from the mice. The spleens were collected for each group containing a control group to prepare single cell suspension. For stimulation in vitro with HSV-1, HSV-1 strain Miyama having a plaque forming unit (PFU) of about 1 X 10^7 was added to 1.25 X 10^8 spleen cells, followed by incubation at 37°C for 1 hour. The stimulated cells were suspended in 50 ml of complete RPMI 1640 medium containing 10% FCS, and cultivated in a plastic flask (Nunc) in the presence of 5% CO^2 at 37°C for 5 days. When the cells were not stimulated with HSV-1, the cultivation was similarly conducted without addition of HSV-1 strain Miyama. After the cultivation, the cells were washed by centrifugation. The number of the viable cells was counted, and then the cells were used as the effector cells.

As the target cells, P388, a macrophage cell line of the BALB/c mouse, was used. 3 X 10^6 P388 cells were incubated with HSV-1 strain Miyama having a PFU of about 3 X 10^6 at 37°C for 1 hour to prepare HSV-1-infected P388 cells. Then, 0.1 mCi sodium chromate solution was added to the HSV-1-infected and non-infected cells to label the cells with ^{51}Cr.

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The spleen cells were added to 1 X 10⁴ ⁵¹Cr-labeled P388 cells so as to give an effector cells/target cells ratio (E/T ratio) of 25 to 100, followed by cultivation on a U-type 96-well microplate (Nunc) at 37°C for 4 hours. The killer activity was calculated from the amount of ⁵¹Cr liberated in the supernatant (200 μl). The determination was carried out twice, and the result was indicated by the mean value of the two determinations. Further, the HSV-1 specific ⁵¹Cr-release (%) was calculated from the following equation:

HSV-1 Specific ^{51}Cr-Release (%) = [^{51}Cr Release from HSV-1-Infected P388 Cells (%)] -[^{51}Cr-Release from HSV-1-Uninfected P388 Cells(%)]

The results are shown in Table 3. The HSV-1-specific and nonspecific killer activities were only observed when the spleen cells of mice to which t-gD-IL-2 was administered was stimulated in vitro with HSV-1. This shows that the cellular immunity to HSV-1 is induced by the administration of t-gD-IL-2.

Table 3

Induction of HSV-1 Specific and Non-specific Killer Activities by Administration of t-gD-IL-2					
Administration in Vivo	HSV-1 Stimulation	HSV-1 Infection of Target	% ^{51}Cr Release		
			E/T Ratio		
			25	50	100
Control	-	-	<1	<1	<1
		+	<1	<1	<1
	+	-	<1	<1	<1
		+	<1	<1	<1
t-gD	-	-	<1	<1	ND[a]
		+	<1	<1	<1
	+	-	<1	<1	ND
		+	<1	<1	<1
t-gD + rIL-2	-	-	<1	<1	<1
		+	<1	<1	<1
	+	-	ND	ND	ND
		+	<1	<1	ND
t-gD-IL-2	-	-	<1	<1	<1
		+	<1	<1	<1
	+	-	14.7	19.7	24.8
		+	26.5 (11.8)[b]	34.3 (14.6)	38.7 (13.9)

a. ND: Not done
b. HSV-1 specific ⁵¹Cr releasing amount

(4) Protection against HSV-1 Challenge

Mice were immunized with each of 1 μg of t-gD, 1 μg of t-gD-IL-2 and mixture of 1 μg of t-gD and 0.25 μg of rIL-2, and protection against HSV-1 challenge in those mice was examined. Namely, each of the above antigens was administered to 8-week-old female BALB/c mice (a group consisting of 6 to 7 mice) in the manner described in the above item (1). After 5 weeks, 0.1 ml/mouse of HSV-1 (Miyama + GC strain) having a PFU of 2 X 10⁵ was intraperitoneally inoculated in the mice. After inoculation, observations were carried out for 17 days to determine the survival ratio of the mice. The results are shown in Fig. 15. The

figures in parentheses indicated the number of the mice used and the number of mice in which the symptoms due to HSV-1 infection were observed (the symptoms appeared in the mice or the mice died).

In the control group (PBS) and the t-gD administration groups, the symptoms due to HSV-1 infection were observed in all mice. Even in the group (mixed) to which the mixture of t-gD and rIL-2 was administered, about half of the mice died. In contrast, in the t-gD-IL-2 administration group (fused), only one mouse died 11 days after the HSV-1 inoculation.

These results show the effect of IL-2 addition to t-gD not only in antibody production, but also in protection against HSV-1 challenge.

Example 14

Preparation of Hybrid Protein Composed of HSV-1 Type Truncated gD and rIL-2

(1) Maleimidation of HSV-1 Truncated gD

1 mg of the HSV truncated gD obtained in Example 11 was dissolved in 2 ml of 5 mM acetate buffer (pH 5.0), and then 50 μl of a bimolar N-(ε-maleimidocaproyloxy)succinimide ester solution in dimetylformamide was added thereto, followed by reaction at 30°C for 20 minutes. The reaction mixture was subjected to a Sephadex G-25 column equilibrated with 0.1 M phosphate buffer (PB, pH 6.5) to remove the combined reagent.

(2) Sulfhydrylation of IL-2

1 mg of the rIL-2 prepared in Japanese Patent Unexamined Publication No. 61-63282/1986 was dissolved in 0.05 M PBS (pH 7.3), and then 50 μl of a bimolar SPDf solution in methanol was added thereto, followed by reaction at 30°C for 30 minutes. After reduction by addition of 50 μl of 0.1 M aqueous solution of DTT, the resulting product was subjected to the Sephadex G-25 column described in the above item (1) to remove the excessive reagent.

(3) Preparation of Antigen-IL-2 Hybrid Protein

0.8 ml of the sulfhydrylated IL-2 prepared in the above item (2) was slowly added to 0.8 mg of the maleimidated truncated gD antigen obtained in the above item (1), with stirring under ice cooling, followed by reaction overnight. The reaction mixture was subjected to a Sephacryl S-200 column to separate and remove unreacted proteins from a chemically combined hybrid protein. As a result, about 1.2 mg of the hybrid protein composed of truncated gD and rIL-2 which were chemically combined with each other was obtained.

Example 15

Construction of Gene Expression Plasmid for Fused Protein Composed of Truncated gpI of VZV (Kizuhara Strain) and IL-2

The plasmid pHDLneoI which was constructed in Example 7 was partially digested with restriction enzyme NheI, and a DNA fragment of about 7.9 kb which was cleaved only at one site of two NheI sites was isolated. The terminus thereof was changed to a flush end with T4 DNA polymerase. Then, the resulting fragment was digested with NheI again, and a portion of the promoter and the gD region were removed to isolate a residual fragment (fragment (1)).

A fragment which was obtained by digesting the plasmid pUC18gpISma containing the VZVgpI gene (Reference Example 2-(3)-ii) with restriction enzyme XbaI was rendered flush with Klenow DNA polymerase, and then inserted into a vector which was obtained by digesting pTB701 (Reference Example 2-(3)-iii) with

restriction enzyme EcoRI, followed by rendering it flush with Klenow DNA polymerase. Thus, gpI expression plasmid pTBgpISma18 was constructed (Fig. 18). This plasmid was digested with restriction enzyme Eco52I to isolate a fragment coding for the amino acid sequence up to the 515th of gpI, and the termini thereof were changed to flush ends with T4 DNA polymerase. The resulting fragment was digested with restriction
5   enzyme BglII to isolate a 1.04-kb fragment (fragment (2)).

Similarly, pTBgpISma18 was digested with NheI and BglII to isolate a fragment of about 2.1 kb containing a portion of the promoter and a portion of gpI (fragment (3)).

The above three fragments (1), (2) and (3) were ligated to one another with T4 DNA ligase to obtain gene expression plasmid pVGL4 for the fused protein composed of VZV truncated gpI and IL-2 (Fig. 18).
10   Further, a fragment (containing an ASVLTR promoter) which was obtained by digesting the truncated expression plasmid pTBE7dhfr4 having the hamster dihydrofolate reductase (hDHFR) as a selected marker (Reference Example 2-(4)) with NheI and HindIII, a fragment (containing an hDHFR gene) which was obtained by digesting the plasmid pTBE7dhfr4 with HindIII and SalI, and a fragment which was obtained by digesting pVGL4 with NheI and SalI were ligated to one another with T4 DNA ligase to construct plasmid
15   pVGLdhfr11 (Fig. 19).

Example 16

20

Gene Expression of Fused Protein Composed of VZV Truncated gpI and Il-2 in COS-7 Cell

The plasmids pVGL4 and pvGLdhfr11 which were constructed in Example 15 were introduced into COS-7 cells to examine transient expression.
25   COS-7 cells (5 X 10^5 cells /10 cm dish) were inoculated into 10 ml of Dulbecco's MEM medium (Gibco) containing 10% FCS, and after 18 hours, the cells were transfected with the above plasmids (20 μg/dish) in accordance with the method of Wigler et al. [Cell 16, 777-785 (1979)]. After 24 hours, the resulting cells were incubated on Dulbecco's MEM medium (Gibco) containing 25 mM HEPES (Donin Chemical Labora-tory for 2 days. Then, 5 ml of the culture supernatant was concentrated to about 200 μl by a Centricut
30   (Centricut 20, Kurabo Industries). 10 μl of this supernatant was mixed with 5 μl of Laemmli buffer having a 3-fold concentration [final concentrations: 62.5 mM Tris-HCl (pH 8.0), 2% SDS, 10% glycerol, 5% 2-ME, 0.001% BPB], and the mixture was heated at 95°C for 5 minutes. This sample was subjected to electrophoresis using 10%-20% SDS polyacrylamide gels (Daiichi Kagaku). After electrophoresis, the sample was assayed by the Western blotting method using a mouse anti-gpI monoclonal antibody (which
35   was obtained from a hybridoma prepared by fusing a spleen cell of a BALB/c mouse immunized with a supernatant of VZV-infected cells disrupted by ultrasonication as an immunogen and mouse myeloma cell SP2 with polyethylene glycol) and a rabbit anti-IL-2 antibody (Genzyme). As a result, in the supernatants of the cells into which pVGL4 and pvGLdhfrII were introduced, a band was detected for each of the ant-gpI antibody and the anti-IL-2 antibody (Fig. 20). On the contrary, in the supernatants of the cells as a control
40   into which pTBE7dhfr4 and pTBgpIEcT (Reference Example 2-(3)-iii) were introduced, a band was only detected for the anti-gpI antibody.

Further, IL-2 biological activity in each supernatant was examined in accordance with the method of Tada et al. [J. Immunol. Methods 93 , 157-165 (1986)]. As a consequence, only when pVGL4 and pVGLdhfr11 were introduced, the IL-2 activity was observed in the supernatant. This revealed that IL-2
45   which was fused with gpI had the biological activity (Table 4).

Table 4

Plasmid	IL-2 Activity (U/ml)
Control	Not detected
pVGL4	0.27
pTBE7dhfr4	Not detected
pVGLdhfr11	0.14
pTBgpIEcT	Not detected

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55

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Example 17

Construction of Gene Expression Plasmid for Fused Protein Composed of Human Immunodeficiency Virus

5 (HIV) gag Protein and IL-2

(1) An SalI linker is added to a 5.1-kb AccII-SalI fragment containing the gag-pol region of HIV recombinant proviral clone pNL4-3 [Adachi et al., J. Virol. 59 , 284-291 (1986); Gen'Bank R62.0 December 1989, locus HIVNL43], and then the resulting fragment is inserted into the SalI site of pBR322 to prepare

10 plasmid pTB770.

(2) The plasmid pTB770 is digested with restriction enzyme XmnI to isolate a 0.43-kb fragment. This fragment is cleaved with BamHI, and then the cleaved fragment is inserted into pUC8 whose termini are changed to flush ends with T4 DNA polymerase to obtain subclone pUC8Xm3.

The subclone pUC8Xm3 is digested with EcoRI and EcoT22I to isolate a 0.42-kb fragment (fragment

15 (1)).

The plasmid pTB770 is digested with BglII, and then the termini of the digested fragment is changed to flush ends with T4 DNA polymerase, followed by digestion with EcoT22I to isolate a 0.85-kb fragment (fragment (2)).

Plasmid pTB505 [Sasada et al., Cell Structure and Function 13 , 129-141 (1988)] for secretory

20 expression of EGF with the signal sequence of IL-2 is digested with EcoRI and SalI to isolate a 1.9-kb fragment (fragment (3)).

The plasmid pHDLneo1 (refer to Example 5) is digested with NheI, and then the termini of the digested fragment is changed to flush ends with T4 DNA polytmerase, followed by digestion with SalI to isolate a 3.0-kb fragment (fragment (4)).

25 The above four fragments (1), (2), (3) and (4) are ligated to one another with T4 DNA ligase to obtain expression plasmid pGAL2 to which genes each coding for the IL-2 signal sequence (containing the amino acid sequence up to Gln^{11}), Ile^{19} to Ile^{437} of the HIV gag protein and Ala^1 to Thr^{133} of IL-2 are ligated downstream from an A-MuLV LTR SV40 promoter (Fig. 21).

(3) Further, in order to modify the plasmid which is obtained in (2) to a stable expression plasmid, the

30 neo gene of the plasmid pHDLneo1 is inserted into pGAL2.

The plasmid pHDLneo1 is digested with ClaI and SalI to isolate a 2.8-kb fragment. This fragment, a 3.6-kb fragment which is obtained by digesting pGAL2 with ClaI and BglII, and a 2.5-kb fragment which is obtained by digesting pGAL2 with SalI and BelII are ligated to one another with T4 DNA ligase to obtain expression plasmid pGALneo (Fig. 22).

35 With respect to the plasmid obtained according to the above methods, the biological activity of the expressed product can be assayed in the same manner as Example 16. The antigenicity of the expressed product can be confirmed by Western blotting using an anti-gag antibody (Chemicon).

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof that will be suggested to persons skilled in the art

40 are to be included in the spirit and purview of this application and the scope of the approved claims.

Claims

45 1. A fused protein comprising an antigen used for vaccine and a lymphokine.

2. A fused protein in accordance with claim 1, in which the antigen used for vaccine is fused with the lymphokine through a linker.

3. A fused protein in accordance with claim 1, in which the lymphokine is interleukin 2.

4. A fused protein in accordance with claim 3, in which the interleukin 2 is human-derived interleukin 2.

50 5. A fused protein in accordance with claim 1, in which the antigen used for vaccine is a viral antigen, a pathogenic protozoal antigen or a pathogenic bacterial antigen.

6. A fused protein in accordance with claim 1, in which the antigen used for vaccine is a herpesvirus antigen.

7. A fused protein in accordance with claim 6, in which the herpesvirus antigen is a herpes simplex virus

55 antigen or a varicella-zoster virus antigen.

8. A fused protein in accordance with claim 1, in which the antigen used for vaccine is a human retrovirus antigen.

9. A fused protein in accordance with claim 8, in which the human retrovirus antigen is a human

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immunodeficiency virus antigen.

10. A fused protein in accordance with claim 1, in which the antigen used for vaccine is a herpes simplex virus surface antigen.

11. A fused protein in accordance with claim 10, in which the herpes simplex virus surface antigen is gD or gB of herpes simplex virus type I or type II.

12. A fused protein in accordance with claim 11, in which the gD or the gB is gD or gB lacking a transmembrane domain.

13. A fused protein in accordance with claim 1, in which a herpes simplex virus surface antigen is arranged on the amino terminal side and interleukin 2 is arranged on the carboxyl terminal side.

14. A recombinant DNA containing a nucleotide sequence coding for a fused protein comprising an antigen used for vaccine and a lymphokine.

15. A recombinant DNA in accordance with claim 14, in which the lymphokine is interleukin 2.

16. A recombinant DNA in accordance with claim 14, in which the antigen used for vaccine is a herpesvirus antigen or a human retrovirus antigen.

17. A recombinant DNA in accordance with claim 14, in which the antigen used for vaccine is a herpes simplex virus surface antigen.

18. A transformant bearing a recombinant DNA containing a nucleotide sequence coding for a fused protein comprising an antigen used for vaccine and a lymphokine.

19. A transformant in accordance with claim 18, in which the lymphokine is interleukin 2.

20. A transformant in accordance with claim 18, in which the antigen used for vaccine is a herpesvirus antigen or a human retrovirus antigen.

21. A transformant in accordance with claim 18, in which the antigen used for vaccine is a herpes simplex virus surface antigen.

22. A method for producing a fused protein comprising an antigen used for vaccine and a lymphokine, the method comprising cultivating a transformant bearing a recombinant DNA containing a nucleotide sequence coding for the fused protein, producing and accumulating the fused protein in a culture, and collecting the fused protein.

23. A method in accordance with claim 22, in which the lymphokine is interleukin 2.

24. A method in accordance with claim 22, in which the antigen used for vaccine is a herpesvirus antigen or a human retrovirus antigen.

25. A method in accordance with claim 22, in which the antigen used for vaccine is a herpes simplex virus surface antigen.

26. A hybrid protein obtained by chemically combining an antigen used for vaccine and a lymphokine.

27. A hybrid protein in accordance with claim 26, in which the antigen used for vaccine is a viral antigen, a pathogenic protozoal antigen or a pathogenic bacterial antigen.

28. A hybrid protein in accordance with claim 26, in which the antigen used for vaccine is a herpesvirus antigen.

29. A hybrid protein in accordance with claim 26, in which the antigen used for vaccine is a herpes simplex virus surface antigen.

30. A hybrid protein in accordance with claim 26, in which the lymphokine is interleukin 2.

31. A fused protein comprising an antigen used for vaccine and a lymphokine in a pharmaceutically acceptable carrier.

32. A pharmaceutical composition which comprises a hybrid protein obtained by chemically combining an antigen used for vaccine with a lymphokine, and a pharmaceutically acceptable carrier.

33. A method for preparing a recombinant DNA containing a nucleotide sequence coding for a fused protein comprising an antigen used for vaccine and a lymphokine, the method comprising inserting the nucleotide sequence into a vector.

34. A method for preparing a transformant bearing a recombinant DNA containing a nucleotide sequence coding for a fused protein comprising an antigen used for vaccine and a lymphokine, the method comprising transforming a microorganism with the recombinant DNA.

35. A method for producing a hybrid protein of an antigen used for vaccine with a lymphokine, which comprises

(a) condensing a reactive amino group of one of the proteins with a reactive carboxyl group of the other protein, or

(b) maleimidating one of the proteins, and reacting the maleimidated protein with the other protein into which a sulfhydryl group is introduced, or

(c) combining one of the proteins having a reactive amino group with the other protein having a reactive amino group by using a dialdehyde reagent, or

21

EDTA : Ethylenediaminetetraacetic acid

SDS : Sodium dodecyl sulfate

DTT : Dithiothreitol

Gly : Glycine (G)

5 Ala : Alanine (A)

Val : Valine (V)

Leu : Leucine (L)

Ile : Isoleucine (I)

Ser : Serine (S)

10 Thr : Threonine (T)

Cys : Cysteine (C)

1/2 Cys: Half cysteine

Met : Methionine (M)

Glu : Glutamic acid (E)

15 Asp : Aspartic acid (D)

Lys : Lysine (K)

Arg : Arginine (R)

His : Histidine (H)

Phe : Phenylalanine (F)

20 Tyr : Tyrosine (Y)

Trp : Tryptophan (W)

Pro : Proline (P)

Asn : Asparagine (N)

Gln : Glutamine (Q)

25 Ap^r : Ampicillin-resistant gene

Tc^r : Tetracycline-resistant gene

ARS 1: Autonomous replication sequence 1

With respect to the proteins of the present invention, a portion of the amino acid sequence may be modified, namely there may be addition, elimination or substitution by a different amino acid(s) as long as

30 the immunogenicity is not lost.

The present invention will hereinafter be described in detail with the following Reference Examples and Examples. It is understood of course that these Reference Examples and Examples are merely illustrative and are not intended to limit the scope of the invention.

Transformant CHO-HDL-1-5 obtained in Example 3 described below and bearing plasmid pHDLdhfr1

35 was deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan (FRI) under the accession number FERM BP-2506 on July 7, 1989. This microorganism was also deposited with the Institute for Fermentation, Osaka, Japan (IFO) under the accession number IFO 50192 on June 26, 1989.

Transformant Escherichia coli DH1/pHSD BJ-1 bearing plasmid PHSD BJ-1 described in Reference

40 Example mentioned below was deposited with the FRI under the accession number FERM BP-1784 on March 9, 1988. This microorganism was also deposited with the IFO under the accession number IFO 14730 on February 23, 1988.

Transformant Saccharomyces cerevisiae NA74-3A($\overline{p}$)/pGFE213 bearing plasmid pGFE213 described in Example 1 mentioned below was deposited with the FRI under the accession number FERM BP-2095 on

45 October 11, 1988. This microorganism was also deposited with the IFO under the accession number IFO 10460 on September 19, 1988.

Animal cell SP-neo-HSD-39 described in Example 6 mentioned below was deposited with the FRI under the accession number FERM BP-2809 on March 16, 1990. This microorganism was also deposited with the IFO under the accession number IFO 50231 on March 1, 1990.

50 Animal cell SP-neo-HDL-245 described in Example 8 mentioned below was deposited with the FRI under the accession number FERM BP-2810 on March 16, 1990. This microorganism was also deposited with the IFO under the accession number IFO 50232 on March 1, 1990.

Transformant Escherichia coli K12 DH1/pTB652 bearing plasmid pTB652 described in Example 5 mentioned below was deposited with the FRI under the accession number FERM BP-1373 on September 5,

55 1986. This microorganism was also deposited with the IFO under the accession number IFO 14539 on August 29, 1986.

Transformant Escherichia coli JM109/pVGL4 bearing plasmid pVGL4 described in Example 15 mentioned below was deposited with the FRI under the accession number FERM BP-2977 on June 20, 1990.

Fig. 1

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  1  Met Gly Gly Ala Ala Ala Arg Leu Gly Ala Val Ile Leu Phe Val Val

 17  Ile Val Gly Leu His Gly Val Arg Gly Lys Tyr Ala Leu Ala Asp Ala

 33  Ser Leu Lys Met Ala Asp Pro Asn Arg Phe Arg Gly Lys Asp Leu Pro

 49  Val Leu Asp Pro Leu Thr Asp Pro Pro Gly Val Arg Arg Val Tyr His

 65  Ile Gln Ala Gly Leu Pro Asp Pro Phe Gln Pro Pro Ser Leu Pro Ile

 81  Thr Val Tyr Tyr Ala Val Leu Glu Arg Ala Cys Arg Ser Val Leu Leu

 97  Asn Ala Pro Ser Glu Ala Pro Gln Ile Val Arg Gly Ala Ser Glu Asp

113  Val Arg Lys Gln Pro Tyr Asn Leu Thr Ile Ala Trp Phe Arg Met Gly

129  Gly Asn Cys Ala Ile Pro Ile Thr Val Met Glu Tyr Thr Glu Cys Ser

145  Tyr Asn Lys Ser Leu Gly Ala Cys Pro Ile Arg Thr Gln Pro Arg Trp

161  Asn Tyr Tyr Asp Ser Phe Ser Ala Val Ser Glu Asp Asn Leu Gly Phe

177  Leu Met His Ala Pro Ala Phe Glu Thr Ala Gly Thr Tyr Leu Arg Leu

193  Val Lys Ile Asn Asp Trp Thr Glu Ile Thr Gln Phe Ile Leu Glu His

209  Arg Ala Lys Gly Ser Cys Lys Tyr Ala Leu Pro Leu Arg Ile Pro Pro

225  Ser Ala Cys Leu Ser Pro Gln Ala Tyr Gln Gln Gly Val Thr Val Asp

241  Ser Ile Gly Met Leu Pro Arg Phe Ile Pro Glu Asn Gln Arg Thr Val

257  Ala Val Tyr Ser Leu Lys Ile Ala Gly Trp His Gly Pro Lys Ala Pro

273  Tyr Thr Ser Thr Leu Leu Pro Pro Glu Leu Ser Glu Thr Pro Asn Ala

289  Thr Gln Pro Glu Leu Ala Pro Glu Asp Pro Glu Asp Ser Ala Leu Leu

305  Glu Asp Pro Val Gly Thr Val Ala Pro Gln Ile Pro Pro Asn Trp His

321  Ile Pro Ser Ile Gln Asp Ala Ala Thr Pro Tyr His Pro Pro Ala Thr

337  Pro Asn Asn Met Gly Leu Ile Ala Gly Ala Val Gly Gly Ser Leu Leu

353  Ala Ala Leu Val Ile Cys Gly Ile Val Tyr Trp Met His Arg Arg Thr

369  Arg Lys Ala Pro Lys Arg Ile Arg Leu Pro His Ile Arg Glu Asp Asp

385  Gln Pro Ser Ser His Gln Pro Leu Phe Tyr
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(d) subjecting both of the proteins into which sulfhydryl groups are introduced to reoxidation reaction.

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Fig. 3-1

                                                                Ala Pro

Ser Ser Pro Gly Thr Pro Gly Val Ala Ala Ala Thr Gln Ala Ala Asn

Gly Gly Pro Ala Thr Pro Ala Pro Pro Ala Pro Gly Pro Ala Pro Thr

Gly Asp Thr Lys Pro Lys Lys Asn Lys Lys Pro Lys Asn Pro Pro Pro

Pro Arg Pro Ala Gly Asp Asn Ala Thr Val Ala Ala Gly His Ala Thr

Leu Arg Glu His Leu Arg Asp Ile Lys Ala lys Asn Thr Asp Ala Asn

Phe Tyr Val Cys Pro Pro Pro Thr Gly Ala Thr Val Val Gln Phe Glu

Gln Pro Arg Arg Cys Pro Thr Arg Pro Glu Gly Gln Asn Tyr Thr Glu

Gly Ile Ala Val Val Phe Lys Glu Asn Ile Ala Pro Tyr Lys Phe Lys

Ala Thr Met Tyr Tyr Lys Asp Val Thr Val Ser Gln Val Trp Phe Gly

His Arg Tyr Ser Gln Phe Met Gly Ile Phe Glu Asp Arg Ala Pro Val

Pro Phe Glu Glu Val Ile Asp Lys Ile Asn Ala Lys Gly Val Cys Arg

Ser Thr Ala Lys Tyr Val Arg Asn Asn Leu Glu Thr Thr Ala Phe His

Arg Asp Asp His Glu Thr Asp Met Glu Leu Lys Pro Ala Asn Ala Ala

Thr Arg Thr Ser Arg Gly Trp His Thr Thr Asp Leu Lys Tyr Asn Pro

Ser Arg Val Glu Ala Phe His Arg Tyr Gly Thr Thr Val Asn Cys Ile

Val Glu Glu Val Asp Ala Arg Ser Val Tyr Pro Tyr Asn Glu Phe Val

Leu Ala Thr Gly Asp Phe Val Tyr Met Ser Pro Phe Tyr Gly Tyr Arg

Glu Gly Ser His Thr Glu His Thr Ser Tyr Ala Ala Asp Arg Phe Lys

Gln Val Asp Gly Phe Tyr Ala Arg Asp Leu Thr Thr Lys Ala Arg Ala

Thr Ala Pro Thr Thr Arg Asn Leu Leu Thr Thr Pro Lys Phe Thr Val

Ala Trp Asp Trp Val Pro Lys Arg Pro Ser Val Cys Thr Met Thr Lys

Trp Gln Glu Val Asp Glu Met Leu Arg Ser Glu Tyr Gly Gly Ser Phe

Arg Phe Ser Ser Asp Ala Ile Ser Thr Thr Phe Thr Thr Asn Leu Thr

Glu Tyr Pro Leu Ser Arg Val Asp Leu Gly Asp Cys Ile Gly Lys Asp

Ala Arg Asp Ala Met Asp Arg Ile Phe Ala Arg Arg Tyr Asn Ala Thr

His Ile Lys Val Gly Gln Pro Gln Tyr Tyr Leu Ala Asn Gly Gly Phe

Leu Ile Ala Tyr Gln Pro Leu Leu Ser Asn Thr Leu Ala Glu Leu Tyr

Fig. 3-2

Val Arg Glu His Leu Arg Glu Gln Ser Arg Lys Pro Pro Asn Pro Thr
Pro Pro Pro Pro Gly Ala Ser Ala Asn Ala Ser Val Glu Arg Ile Lys
Thr Thr Ser Ser Ile Glu Phe Ala Arg Leu Gln Phe Thr Tyr Asn His
Ile Gln Arg His Val Asn Asp Met Leu Gly Arg Val Ala Ile Ala Trp
Cys Glu Leu Gln Asn His Glu Leu Thr Leu Trp Asn Glu Ala Arg Lys
Leu Asn Pro Asn Ala Ile Ala Ser Val Thr Val Gly Arg Arg Val Ser
Ala Arg Met Leu Gly Asp Val Met Ala Val Ser Thr Cys Val Pro Val
Ala Ala Asp Asn Val Ile Val Gln Asn Ser Met Arg Ile Ser Ser Arg
Pro Gly Ala Cys Tyr Ser Arg Pro Leu Val Ser Phe Arg Tyr Glu Asp
Gln Gly Pro Leu Val Glu Gly Gln Leu Gly Glu Asn Asn Glu Leu Arg
Leu Thr Arg Asp Ala Ile Glu Pro Cys Thr Val Gly His Arg Arg Tyr
Phe Thr Phe Gly Gly Gly Tyr Val Tyr Phe Glu Glu Tyr Ala Tyr Ser
His Gln Leu Ser Arg Ala Asp Ile Thr Thr Val Ser Thr Phe Ile Asp
Leu Asn Ile Thr Met Leu Glu Asp His Glu Phe Val Pro Leu Glu Val
Tyr Thr Arg His Glu Ile Lys Asp Ser Gly Leu Leu Asp Tyr Thr Glu
Val Gln Arg Arg Asn Gln Leu His Asp Leu Arg Phe Ala Asp Ile Asp
Thr Val Ile His Ala Asp Ala Asn Ala Ala Met Phe Ala Gly Leu Gly
Ala Phe Phe Glu Gly Met Gly Asp Leu Gly Arg Ala Val Gly Lys Val
Val Met Gly Ile Val Gly Gly Val Val Ser Ala Val Ser Gly Val Ser
Ser Phe Met Ser Asn Pro Phe Gly Ala Leu Ala Val Gly Leu Leu Val
Leu Ala Gly Leu Ala Ala Ala Phe Phe Ala Phe Arg Tyr Val Met Arg
Leu Gln Ser Asn Pro Met Lys Ala Leu Tyr Pro Leu Thr Thr Lys Glu
Leu Lys Asn Pro Thr Asn Pro Asp Ala Ser Gly Glu Gly Glu Glu Gly
Gly Asp Phe Asp Glu Ala Lys Leu Ala Glu Ala Arg Glu Met Ile Arg
Tyr Met Ala Leu Val Ser Ala Met Glu His Thr Glu His Lys Ala Lys
Lys Lys Gly Thr Ser Ala Leu Leu Ser Ala Lys Val Thr Asp Met Val
Met Arg Lys Arg Arg Asn Thr Asn Tyr Thr Gln Val Pro Asn Lys Asp
Ser Asp Ala Asp Glu Asp Asp Leu

Fig. 4-1

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1                                                      GAGTTGCGCCGCCCG    15

GACTGCAGCCGCCCGACCTCCGAAGGTCGTTACCGTTACCCGCCCGGCGTAT            67

ATCTCACGTACGACTCCGACTGTCCGCTGGTGGCCATCGTCGAGAGCGCCCC           119

CGACGGCTGTATCGGCCCCCGGTCGGTCGTGGTCTACGACCGAGACGTTTTC          171

TCGATCCTCTACTCGGTCCTCCAGCACCTCGCCCCCAGGCTACCTGACGGGG          223

 GGCACGACGGGCCCCCGTAGTCCCGCC ATG CGC CAG GGC GCC CCC GCG   271

CGG GGG TGC CGG TGG TTC GTC GTA TGG GCG CTC TTG GGG TTG ACG   316
                              ↓
CTG GGG GTC CTG GTG GCG TCG GCG GCT CCG AGT TCC CCC GGC ACG   361

CCT GGG GTC GCG GCC GCG ACC CAG GCG GCG AAC GGG GGA CCT GCC   406

ACT CCG GCG CCG CCC GCC CCT GGC CCC GCC CCA ACG GGG GAC ACG   451

AAA CCG AAG AAG AAC AAA AAA CCG AAA AAC CCA CCG CCG CCG CGC   496

CCC GCC GGC GAC AAC GCG ACC GTC GCC GCG GGC CAC GCC ACC CTG   541

CGC GAG CAC CTG CGG GAC ATC AAG GCG AAG AAC ACC GAT GCA AAC   586

TTT TAC GTG TGC CCA CCC CCC ACG GGC GCC ACG GTG GTG CAG TTC   631

GAG CAG CCG CGC CGC TGC CCG ACC CGG CCC GAG GGT CAG AAC TAC   676

ACG GAG GGC ATC GCG GTG GTC TTC AAG GAG AAC ATC GCC CCG TAC   721

AAG TTC AAG GCC ACC ATG TAC TAC AAA GAC GTC ACC GTT TCG CAG   766

GTG TGG TTC GGC CAC CGC TAC TCC CAG TTT ATG GGG ATC TTT GAG   811

GAC CGC GCC CCC GTC CCC TTC GAG GAG GTG ATC GAC AAG ATC AAC   856

GCC AAG GGG GTC TGT CGG TCC ACG GCC AAG TAC GTG CGC AAC AAC   901

CTG GAG ACC ACC GCG TTT CAC CGG GAC GAC CAC GAG ACC GAC ATG   946

GAG CTG AAA CCG GCC AAC GCC GCG ACC CGC ACG AGC CGG GGC TGG   991

CAC ACC ACC GAC CTC AAG TAC AAC CCC TCG CGG GTG GAG GCG TTC   1036

CAC CGG TAC GGG ACG ACG GTA AAC TGC ATC GTC GAG GAG GTG GAC   1081

GCG CGC TCG GTG TAC CCG TAC AAC GAG TTT GTG CTG GCG ACT GGC   1126

GAC TTT GTG TAC ATG TCC CCG TTT TAC GGC TAC CGG GAG GGG TCG   1171

CAC ACC GAA CAC ACC AGC TAC GCC GCC GAC CGC TTC AAG CAG GTC   1216

GAC GGC TTC TAC GCG CGC GAC CTC ACC ACC AAG GCC CGG GCC ACG   1261
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Fig. 4-2

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GCG CCG ACC ACC CGG AAC CTG CTC ACG ACC CCC AAG TTC ACC GTG  1306
GCC TGG GAC TGG GTG CCA AAG CGC CCG TCG GTC TGC ACC ATG ACC  1351
AAG TGG CAG GAG GTG GAC GAG ATG CTG CGC TCC GAG TAC GGC GGC  1396
TCC TTC CGA TTC TCC TCC GAC GCC ATA TCC ACC ACC TTC ACC ACC  1441
AAC CTG ACC GAG TAC CCG CTC TCG CGC GTG GAC CTG GGG GAC TGC  1486
ATC GGC AAG GAC GCC CGC GAC GCC ATG GAC CGC ATC TTC GCC CGC  1531
AGG TAC AAC GCG ACG CAC ATC AAG GTG GGC CAG CCG CAG TAC TAC  1576
CTG GCC AAT GGG GGC TTT CTG ATC GCG TAC CAG CCC CTT CTC AGC  1621
AAC ACG CTC GCG GAG CTG TAC GTG CGG GAA CAC CTC CGA GAG CAG  1666
AGC CGC AAG CCC CCA AAC CCC ACG CCC CCG CCG CCC GGG GCC AGC  1711
GCC AAC GCG TCC GTG GAG CGC ATC AAG ACC ACC TCC TCC ATC GAG  1756
TTC GCC CGG CTG CAG TTT ACG TAC AAC CAC ATA CAG CGC CAT GTC  1801
AAC GAT ATG TTG GGC CGC GTT GCC ATC GCG TGG TGC GAG CTG CAG  1846
AAT CAC GAG CTG ACC CTG TGG AAC GAG GCC CGC AAG CTG AAC CCC  1891
AAC GCC ATC GCC TCG GTC ACC GTG GGC CGG CGG GTG AGC GCG CGG  1936
ATG CTC GGC GAC GTG ATG GCC GTC TCC ACG TGC GTG CCG GTC GCC  1981
GCG GAC AAC GTG ATC GTC CAA AAC TCG ATG CGC ATC AGC TCG CGG  2026
CCC GGG GCC TGC TAC AGC CGC CCC CTG GTC AGC TTT CGG TAC GAA  2071
GAC CAG GGC CCG TTG GTC GAG GGG CAG CTG GGG GAG AAC AAC GAG  2116
CTG CGG CTG ACG CGC GAT GCG ATC GAG CCG TGC ACC GTG GGA CAC  2161
CGG CGC TAC TTC ACC TTC GGT GGG GGC TAC GTG TAC TTC GAG GAG  2206
TAC GCG TAC TCC CAC CAG CTG AGC CGC GCC GAC ATC ACC ACC GTC  2251
AGC ACC TTC ATC GAC CTC AAC ATC ACC ATG CTG GAG GAT CAC GAG  2296
TTT GTC CCC CTG GAG GTG TAC ACC CGC CAC GAG ATC AAG GAC AGC  2341
GGC CTG CTG GAC TAC ACG GAG GTC CAG CGC CGC AAC CAG CTG CAC  2386
GAC CTG CGC TTC GCC GAC ATC GAC ACG GTC ATC CAC GCC GAC GCC  2431
AAC GCC GCC ATG TTC GCG GGC CTG GGC GCG TTC TTC GAG GGG ATG  2476
GGC GAC CTG GGG CGT GCG GTC GGC AAG GTG GTG ATG GGC ATC GTG  2521
```

Fig. 4-3

GGC GGC GTG GTA TCG GCC GTG TCG GGC GTG TCC TCC TTC ATG TCC 2566

AAC CCC TTT GGG GCG CTG GCC GTG GGT CTG TTG GTC CTG GCC GGC 2611

CTG GCG GCG GCC TTC TTC GCC TTT CGC TAC GTC ATG CGG CTG CAG 2656

AGC AAC CCC ATG AAG GCC CTG TAC CCG CTA ACC ACC AAG GAG CTC 2701

AAG AAC CCC ACC AAC CCG GAC GCG TCC GGG GAG GGC GAG. GAG GGC 2746

GGC GAC TTT GAC GAG GCC AAG CTA GCC GAG GCC CGG GAG ATG ATA 2791

CGG TAC ATG GCC CTG GTG TCG GCC ATG GAG CAC ACG GAA CAC AAG 2836

GCC AAG AAG AAG GGC ACG AGC GCG CTG CTT AGC GCC AAG GTC ACC 2881

GAC ATG GTC ATG CGC AAG CGC CGC AAC ACC AAC TAC ACC CAA GTT 2926

Stop
CCC AAC AAA GAC AGT GAC GCC GAC GAG GAC GAC CTG TGA CGGGGG 2971

GTTTGTTGTAAATAAAAACCACGGGTGTTAAACCGCATGCGCATCTTTTGGT 3023

TTTTTTTTTTGTACGCCCTTTGTGTGTGTGGGAAGAAAGAAAAAGGAACACA 3075

TAAACTCCCCCGGGTGTCCGCGGCCTGTTTCCTCTTTCCTTTCCCGTGACAA 3127

AACTGACCCCCTTGGTCAGTGCCGATTCCCCCCCCCCCCCCCACGCCTTCCT 3179

CCACGTCGAAGGCTTTTGTATTGTAAAGCTACCCGCCTACCCGCGCCTCCCA 3231

ATAAAAAAAAAAAGAACATACACCAATGGGTCTTATTTGGTATTACCTGGTT 3283

TATTTAAAAAGATATACAGTAAGACATCCCATGGTACCAAAGACCGGGGCGA 3335

ATCAGCGGGCCCCCATCATCTGAGAGACGAACAAATCGGCGGCGCGGGCCGT 3387

GTCAACGTCCACGTGTGCTGCGCTGCTGGCGTTGACAAGGGCCCCGGCCTCC 3439

GCGTTGGATGCCTCCGGTTGGGATCC 3465

Fig. 5-1

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  1-GTCAACGGGCCCCTCTTTGATCACTCCACCACAGCTTCGCCCAACACCGGCTGTATTACAGGCTGCGAGAACGTGGGGCTCCTGCCGCACC

101-TGAAGGAGGAGCTCGCCCGGTTCATCATGGGGGGCTCGGGTGCTGATTGGGCCGTCAGGGAATTCAGAGGTTTACTGTTTGACGGCATTTC

201-CGGAATAACGCCCACTCAGCGCGCCGCGCTGGCGATATATTCGGCAGCTGATTATCGCCACCTCTTTGCCTCGGTCTCACCGGTGCGGGAGCTCGAG

301-TTGCGCCGCCCGGACTGCAGCCCGGACCTCCGAAGGTCGTTACCGTTACCCGGGCGTATATCTCACGTACGACTCGGACGTGTCCGCTGGTGGCCA

401-TCGTCGAGAGGAGGCCCCGACGGGTGTATCGGCCCCGGTCGTGGTGGTCTACGACGCCCGGTCGTCCGGCGTCGTTCTTCTACTCGGTCGTCCTCCAGCACCTCGC

501-CCCCAGGCTACCTGACGGGGGCACGACGGGCCCCGTAGTCCCGCC ATG CAC CAG GGC GCC CCC TCG TGG GGG CGC GGG TGG TTC
                                                Met His Gln Gly Ala Pro Ser Trp Gly Arg Arg Trp Phe-13

587-GTC GTA TGG GCG CTC TTG GGG TTG ACG CTG GGG GTC CTG GTG GCG TCG GCT AGT TCC CCC GGC ACG CCT
    Val Val Trp Ala Leu Leu Gly Leu Thr Leu Gly Val Leu Val Ala Ser Ala Pro Ser Pro Gly Thr Pro-38

662-GGG GTC GGC GAC CCA CGC GGC GAA CGG GGC GGG CCC TGC GGC GCC GCC CTT GGC GCC GCA CTG GCC CCA ACG
    Gly Val Gly Asp Pro Arg Gly Glu Arg Gly Gly Pro Cys Gly Arg Gly Ala Ala Leu Gly Ala Ala Pro Ala Pro Thr-63

737-GGG GAC CCG AAA CCG AAG AAG AAC AAA AAC CCA ACG CCA CGC GCG ATC CGG GAG CAC CTG CGC GAG
    Gly Asp Pro Lys Pro Lys Lys Asn Lys Asn Pro Thr Pro Arg Ala Ile Arg Glu His Leu Arg Glu  Asn Ala Thr-88

812-GTC GCC GCG CAC GCC ACC CTG GAG GTG GCC GGC ATC GAG AAC ACC GAT GCA AAC TTT TAC
    Val Ala Ala Gly His Ala Thr Leu Arg Glu Asp Ala Gly Ile Lys Ala Glu Asn Thr Asp Ala Asn Phe Tyr-113

887-GTG TGC CCA CCC CCC ACG GGC GCC GTG GTG ACG GTC CCG ACC CGG GAG GGT
    Val Cys Pro Pro Pro Thr Gly Ala Thr Val Val Gln Pro Gln Arg Arg Pro Thr Arg Pro Glu Gly Gly-138

962-CAG AAC TAC ACG GAG GGC ATC GAG GTC TTC GTG GTC AAG AAC ATC GCC CCG TAC AAG TTC ATG TAC
    Gln Asn Tyr Thr Glu Gly Ile Glu Ala Val Val Phe Lys Glu Val Asn Ile Ala Pro Tyr Lys Phe Ala Thr Met Tyr-163

1037-TAC AAA GAC GTC ACC GTT TCG CAG TGG TTC CAG TTT ATG GGG ATC TTT GAG GAC CGC
     Tyr Lys Asp Val Thr Val Ser Gln Val Trp Phe Gly His Arg Tyr Ser Gln Phe Met Gly Ile Phe Glu Asp Arg-188

1112-GCC CCC GTC CCC TTC GAG ATC AAC GAC AAG ATC AAC GCC AAG GGG GTC TCC ACG TCC GTC GTG TAC GTG
     Ala Pro Val Pro Phe Glu Ile Asn Asp Lys Ile Asn Ala Lys Gly Val Cys Arg Ser Thr Ala Lys Tyr Val-213

1187-CGC AAC AAC CTG GAG ACC GAG ATG TTT CAC CGG GAC GAC ACC CCG AAA CCG GCC AAC GCC
     Arg Asn Asn Leu Glu Thr Glu Met Asp His Arg Asp Asp Thr Phe Ala His His Glu Pro Lys Leu Pro Ala Asn Ala-238
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Fig. 5-2

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1262-GCG ACC CGC ACG AGC CGG GGC TGG CAC ACC ACC AAG CTC GAC TAC AAC CCC TCG CGG GTG GAG GCG TTC CAC CGG
     Ala Thr Arg Thr Ser Arg Gly Trp His Thr Thr Lys Leu Asp Tyr Asn Pro Ser Arg Val Glu Ala Phe His Arg-263

1337-TAC GGG ACG ACA AAC GTA TGC ATC GTC GAG GAC GCG CGC TAC GTG TCG CCG GAG TAC ACG TTT GTG CTG
     Tyr Gly Thr Thr Asn Val Cys Ile Val Glu Asp Ala Arg Tyr Val Ser Arg Glu Tyr Pro Phe Val Leu-288

1412-GCG ACT GGC GAC TTT GTG ATC CCG TAC ATG TCC CCG TTT TAC CGG GGG TAC GGG TCG CAC ACC ACC TAC
     Ala Thr Gly Asp Phe Val Ile Pro Tyr Met Ser Pro Phe Tyr Arg Gly Tyr Gly Ser His Thr Thr Tyr-313

1487-GCC GCC GAC CGC AAG CAG GTC GTC TTC GAC CAG GGC GTC GCG GCC TTC GAC GCC AAG GCG GCG CCG CCG
     Ala Ala Asp Arg Lys Gln Val Val Phe Asp Gln Gly Val Ala Ala Phe Asp Ala Lys Ala Ala Pro Pro-338

1562-ACC AAC CGG ACC AAG CTG ACG CCC AAG CCC CGG TGG GTG GCC CCA GTG CGC CCG TCG GTC CCG TCG GTC TGC
     Thr Asn Arg Thr Lys Leu Thr Pro Lys Pro Arg Trp Val Ala Pro Val Arg Pro Ser Val Pro Ser Val Cys-363

1637-ACC ATG ACC AAG TGG CAG GAA GTG GAC ATG GAG CTG TCC GGC TAC TCC TTC TTC TCC GAC AGC TCC GAC
     Thr Met Thr Lys Trp Gln Glu Val Asp Met Glu Leu Ser Gly Tyr Ser Phe Phe Ser Asp Ser Ser Asp-388

1712-GCC ATA TCC ACC ACC TTC ACC ACC AAC CTG ACC AAC CTG ACC CGC CTC TCG TAC CCG CTC TAC GAC ATC GGG
     Ala Ile Ser Thr Thr Phe Thr Thr Asn Leu Thr Asn Leu Thr Arg Leu Ser Tyr Pro Leu Tyr Asp Ile Gly-413

1787-AAG GAC GCC CGC GAC ATG GCC GAC ATC TTC GCC AGG TAC CAC ATC AAG GTG GGC CAG CCG
     Lys Asp Ala Arg Asp Met Ala Asp Ile Phe Ala Arg Tyr His Ile Lys Val Gly Gln Pro-438

1862-CAG TAC TAC CTG GCC AAT GGG GGG TTT CTG ATC GCG TAC CAG CCC CTC AGC AAC ACG CTC GAG CTG TAC
     Gln Tyr Tyr Leu Ala Asn Gly Gly Phe Leu Ile Ala Tyr Gln Pro Leu Ser Asn Thr Leu Glu Leu Tyr-463

1937-GTG CGG GAA CAC CTC GGC GAG CAG CGA AGC CAG GAG CCC ACG AAC CCC CCG ACG CCC GGG GCC AGC AAC
     Val Arg Glu His Leu Gly Glu Gln Arg Ser Gln Glu Pro Thr Asn Pro Pro Thr Pro Gly Ala Ser Asn-488

2012-GCG TCC GTG ACC ATC AAG GCC ACC TCC TCC ATC GAG CAG TTC ACG CGG CTG GCC TAC ACC AAC CAC ATA
     Ala Ser Val Thr Ile Lys Ala Thr Ser Ser Ile Glu Gln Phe Thr Arg Leu Ala Tyr Thr Asn His Ile-513

2087-CGG CAT GTC AAC GAT ATG TTG GGC CTG GGC CGG TGC GAG CTA CAG AAT CAC CAG ATA CTG ACC CTG TGG
     Arg His Val Asn Asp Met Leu Gly Leu Gly Arg Cys Glu Leu Gln Asn His Gln Ile Leu Thr Leu Trp-538

2162-AAC GAG GCC GCC CGG AAG CTG AAC CCC AAC GCC ATC GCC ATC GCC GTC TCG GTG ACC GGC GTG CGG GCG ATG CTC
     Asn Glu Ala Ala Arg Lys Leu Asn Pro Asn Ala Ile Ala Ile Ala Val Ser Val Thr Gly Val Arg Ala Met Leu-563
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F i g. 5 — 3

2237-GGC GAC GTG·ATG GCC GTC TCC ACG TGC GTG CCG GTC GCC GCC AAC GTG ATC GTC CAA AAC TCG ATG CGC ATC
     Gly Asp Val Met Ala Val Ser Thr Cys Val Pro Val Ala Ala Asn Val Ile Val Gln Asn Ser Met Arg Ile-588

2312-AGC TCG CGG CCC TGC TAC AGC CGC CTG GTC AGC TTT CGG TAC GAA GAC CAG GGC TTG GTC GAG
     Ser Ser Arg Pro Gly Ala Cys Tyr Ser Arg Pro Leu Val Ser Arg Tyr Phe Glu Arg Asp Gln Gly Pro Leu Val Glu-613

2387-GGG GAG CTG GGG GAG AAC AAC GAG CTG CGG ACG ATC GCG GAT CGC GTG GGA CAC CGG CGC
     Gly Glu Leu Gly Glu Asn Asn Glu Leu Arg Thr Ile Ala Asp Arg Val Gly His Arg Arg
     Gln Leu Ser Arg Ala Asp Ile Glu Pro Cys Thr Val Gly His Arg Arg-638

2462-TAC TTC ACC TTC GGT GGG TAC TAC GAG GAG TAC CAC CAG CTG AGC GCC GAC ATC
     Tyr Phe Thr Phe Gly Gly Tyr Tyr Glu Glu Tyr His Gln Leu Ser Arg Ala Asp Ile
     Tyr Val Tyr Phe Glu Glu Tyr His Gln Leu Ser Arg Ala Asp Ile-663

2537-ACC ACC GTC ATC TTC GAC CTC AAC ATC ACC GAG GAT CAC TTT GTC CCC CTG TAC
     Thr Thr Val Ser Thr Phe Ile Asp Leu Asn Ile Thr Met Leu Glu Asp His Phe Val Pro Leu Glu Glu Val Tyr-688

2612-ACC CGC CAC GAG ATC AAG GAC AGC GGG CTG CTG GTC TAC GAC GAG GAG CAG AAC CGC CTG
     Thr Arg His Glu Ile Lys Asp Ser Gly Leu Leu Asp Val Tyr Thr Glu Val Gln Gln Asn Arg Arg Leu
     Thr Arg His Glu Ile Lys Asp Ser Gly Leu Leu Asp Val Tyr Thr Glu Val Gln Arg Asn Gln Leu Leu-713

2687-CGC TTC GCC GAC ATC GTC CAC GGG CTG GGC GTG TTC GCG
     Arg Phe Ala Asp Ile Val His Gly Leu Gly Val Phe Ala
     Arg Phe Ala Asp Ile Val His Gly Met Phe Ala Gly Leu Gly Ala Phe Phe-738

2762-GAG GGG ATG GGG GAC GAC CTG GGG GTG GTG TCG GCG
     Glu Gly Met Gly Asp Asp Leu Gly Val Val Ser Ala
     Glu Ala Arg Ala Leu Gly Met Lys Val Val Gly Val Val Ser Ala Val-763

2837-TCG GGG TCC TCC TTC ATG TCC AAC CCC GGG CTG CTG GTC GCC GGC
     Ser Gly Ser Ser Phe Met Ser Asn Pro Gly Leu Leu Val Ala Gly
     Ser Gly Val Ser Phe Met Ser Asn Pro Gly Ala Leu Leu Val Ala Gly Leu Leu Ala Gly Leu Ala-788

2912-GCG GCC TTC TTC GCC AAC AAC CCC ATG TAC CGT CAG AGC TAC CCT CTA ACC ACC
     Ala Ala Phe Phe Ala Asn Asn Pro Met Tyr Arg Gln Ser Tyr Pro Leu Thr Thr
     Ala Ala Phe Phe Ala Arg Asn Pro Met Tyr Val Val Ala Leu Tyr Pro Leu Thr Thr Thr-813

2987-GCG GAG GAG CTC AAG CTC AAG AAC ACC CCG ACC GCC TTT GAC GAG GCC AAG
     Ala Glu Glu Leu Lys Leu Lys Asn Thr Pro Thr Ala Phe Asp Glu Ala Lys
     Ala Glu Glu Leu Lys Leu Lys Asn Ser Gly Gly Gly Glu Phe Asp Glu Ala Lys Ala Lys-838

3062-CTA GCC GAG ATG ATA CGG TAC CTG GCC ATG GTC ACG GAA CAC AAG AAG GCC AAG
     Leu Ala Glu Met Ile Arg Tyr Met Ala Met Val Thr Glu His Lys Lys Ala Lys
     Leu Ala Glu Met Ile Arg Tyr Met Ala Met Val Thr Glu His Lys Lys Ala Lys-863

3137-AAG AAG GGC ACG ACG AGG CGG CTG CTC AGC GCC GCC GTC ACC AAC TAC
     Lys Lys Gly Thr Thr Arg Arg Leu Leu Ser Ala Ala Val Thr Asn Tyr
     Lys Lys Gly Thr Ser Arg Leu Leu Ser Ala Ala Lys Val Thr Asp Met Val Thr Met Asp Met Val Arg Lys Arg Arg Asn Thr Asn Tyr-888

Fig. 5 - 4

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3212-ACC CAA GTT CCC AAC AAA GAC GGT GAC GCC GAC GAG GAC CTG TGACGGGGGGTTTGTTGTAAATAAAAACCACGGGTGTTAA
     Thr Gln Val Pro Asn Lys Asp Gly Asp Ala Asp Glu Asp Leu END

3297-ACCGCATGGCGATCTTTGGTTTTTGTTTGGTCAGCCTTTGTTGTGTGTGGGAAGAAAAAGGAACACATAAACTCCCCGGGTGTCCGCGGC

3397-CTGTTTCCTCTTTCCTTTCCGTGACAAACGGACCCCCTTGGTCAGTGCCGATTTCCTCCCCACGCCTTCCTCCACGTCAAAGGCTTTGCATTGT

3497-AAAGCTACCCGCTACCCGCCCTCCCAATAAAAAAAAGAACATACACCAATGGGTCTTATTTGGTATTACCTGGTTTÅTTTAAAAAGATATACAGTA

3597-AGACATCCCATGGTACCAAAGACCGGGGCGAATCAGCGGCCCCATCATCTGAGAGACGAACAAATCGGCGGCGGGGCCGTGTCAACGTCCACGTGTG

3697-CTGCGCTGCTGGGCGTTGACAAGGCCCCGGCCTCCGCGTTGGATGCTCCGGTTGGGATCC
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Fig. 6-1

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CTCGGAGAAGATGCTGCGGTCAGCGTCCACGGCGAGGTGCTGCCCGCGACGTTCGCCGCG        60

GTCGCCAACGGCTTTGCGGCCGCGCGCCGGCTTCTCGCCGCCCCCTGACGGCGGGGCGCGGG       120

CACGGTCATCGACAACCGCTCGGCGCCGGGCGTGTTCGACGCGCACCGGTTCATGCGAGC        180

GTCTCTCCTGCGACACCAGGTGGACCCGGCCCTGCTCCCCAGCATCACCCATCGCTTCTT       .240

CGAGCTCGTCAACGGGCCCCTCTTTGATCACTCCACCCACAGCTTCGCCCAGCCCCCCAA        300

CACCGCGCTGTATTACAGCGTCGAGAACGTGGGGGCTCCTGCCGCACCTGAAGGAGGAGC.T      360

CGCCCGGTTCATCATGGGGGCGGGGGGCTCGGGTGCTGATTGGGCCGTCAGCGAATTTCA        420

GAGGTTTTACTGTTTTGACGGCATTTCCGGAATAACGCCCACTCAGCGCGCCGCCTGGCG        480

ATATATTCGCGAGCTGATTATCGCCACCACACTCTTTGCCTCGGTCTACCGGTGCGGGGA        540

GCTCGAGTTGCGCCGCCCGGACTGCAGCCGCCCGACCTCCGAAGGTCGTTACCGTTACCC        600

GCCCGGCGTATATCTCACGTACGACTCCGACTGTCCGCTGGTGGCCATCGTCGAGAGCGC        660

CCCCGACGGCTGTATCGGCCCCCGGTCGGTCGTGGTCTACGACCGAGACGTTTTCTCGAT        720

CCTCTACTCGGTCCTCCAGCACCTCGCCCCCCAGGCTACCTGACGGGGGGGCACGACGGGC       779
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                  MetArgGlnGlyAlaAlaArgGlyCysArgTrpPheValValTrp        -15
CCCCGTAGTCCCGCCATGCGCCAGGGCGCCGCGCGGGGGTGCCGGTGGTTCGTCGTATGG          839

AlaLeuLeuGlyLeuThrLeuGlyValLeuValAlaSerAlaAlaProSerSerProGly           6
GCGCTCTTGGGGTTGACGCTGGGGGGTCCTGGTGGCGTCGGCGGCTCCGAGTTCCCCCGGC        899

ThrProGlyValAlaAlaAlaThrGlnAlaAlaAsnGlyGlyProAlaThrProAlaPro          26
ACGCCTGGGGTCGCGGCCGCGACCCAGGCGGCGAACGGGGGACCTGCCACTCCGGCGCCG         959

ProAlaProGlyProAlaProThrGlyAspThrLysProLysLysAsnLysLysProLys          46
CCCGCCCCTGGCCCCTCCCCAACGGGGGACACGAAACCGAAGAACAACAAAAAACCGAAA        1019

AsnProProProProArgProAlaGlyAspAsnAlaThrValAlaAlaGlyHisAlaThr          66
AACCCACCGCCGCCGCGCCCCGCCGGCGACAACGCGACCGTCGCCGCGGGCCACGCCACC        1079

LeuArgGulHisLeuArgAspIleLysAlaGluAsnThrAspAlaAsnPheTyrValCys          86
CTGCGCGAGCACCTGCGGGACATCAAGGCGGACAACACCGATGCAAACTTTTACGTGTGC        1139

ProProProThrGlyAlaThrValValGlnPheGluGlnProArgArgCysProThrArg        106
CCACCCCCCACGGGCGCCACGGTGGTGCAGTTCGACGAGCCGCGCCGCTGCCCGACCCGG        1199

ProGluGlyGlnAsnTyrThrGluGlyIleAlaValValPheLysGluAsnIleAlaPro        126
CCCGAGGGTCAGAACTACACGGAGGGCATCGCGGTGGTCTTCAAGGACAACATCGCCCCG        1259

TyrLysPheLysAlaThrMetTyrTyrLysAspValThrValSerGlnValTrpPheGly        146
TACAAGTTCAAGGCCACCATGTACTACAAAGACGTCACCGTTTCGCAGGTGTGGTTCGGC        1319

HisArgTyrSerGlnPheMetGlyIlePheGluAspArgAlaProValProPheGluGlu        166
CACCGCTACTCCCAGTTTATGGGGATCTTTGAGGACCGCGCCCCCGTCCCCTTCGAGGAG        1379

ValIleAspLysIleAsnAlaLysGlyValCysArgSerThrAlaLysTyrValArgAsn        186
GTGCTCGACAAGATCAACGCCAAGGGGGTCTGTCGGTCCACGGCCAAGTACGTGCGCAAC        1439
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Fig. 6-2

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AsnLeuGluThrThrAlaPheHisArgAspAspHisGluThrAspMetGluLeuLysPro      206
AACCTGGAGACCACCGCGTTTCACCGGGACGACCACGAGACCGACATGGAGCTGAAACCG    1499

AlaAsnAlaAlaThrArgThrSerArgGlyTrpHisThrThrAspLeuLysTyrAsnPro      226
GCCAACGCCGCGACCCGCACGAGCCGGGGCTGGCACACCACCGACCTCAAGTACAACCCC    1559

SerArgValGluAlaPheHisArgTyrGlyThrThrValAsnCysIleValGluGluVal      246
TCGCGGGTGGAGGCGTTCCACCGGTACGGGACGACGGTAAACTGCATCGTCGAGGAGGTG    1619

AspAlaArgSerValTyrProTyrAspGluPheValLeuAlaThrGlyAspPheValTyr      266
GACGCGCGCTCGGTGTACCCGTACGACGAGTTTGTGCTGGCGACTGGCGACTTTGTGTAC    1679

MetSerProOheTyrGlyTyrArgGluAlySerHisThrGluHisThrSerTyrAlaAla      286
ATGTCCCCGTTTTACGGCTACCGGGAGGGGTCGCAGACCGAACACACCAGCTACGCCGCC    1739

AspArgPheLysGlnValAspGlyPheTyrAlaArgAspLeuThrTyrLysAlaArgAla      306
GACCGCTTCAAGCAGGTTGACGGCTTCTACGCGCGCGACCTCACCACCAAGGCCCGGGCC    1799

ThrAlaProThrThrArgAsnLeuLeuThrThrProLysPheThrValAlaTrpAspTrp      326
ACGGCGCCGACCACCCGGAACCTGCTCACGACCCCCAAGTTCACCGTGGCCTGGGACTGG    1859

ValProLysArgProSerValCysThrMetThrLysTrpGlnGluValAspGluMetLeu      346
GTGCCAAAGCGCCCGTCGGTCTGCACCATGACCAAGTGGCAGGAGGTGGACGAGATGCTG    1919

ArgSerGluTyrGlyGlySerPheArgPheSerSerAspAlaIleSerThrThrPheThr      366
CGCTCCGAGTACGGCGGCTCCTTCCGATTCTCCTCCGACGCCATATCCACCACCTTCACC    1979

ThrAsnLeuThrGluTyrProLeuSerArgValAspLeuGlyAspCysIleGlyLysAsp      386
ACCAACCTGACCGAGTACCCGCTCTCGCGCGTGGACCTGGGGGGACTGCATCGGCAAGGAC    2039

AlaArgAspAlaMetAspArgIlePheAlaArgArgTyrAsnAlaThrHisIleLysVal      406
GCCCGCGACGCCATGGACCGCATCTTCGCCCGCAGGTACAACGCGACGCACATCAAGGTG    2099

GlyGlnProGlnTyrTyrLeuAlaAsnGlyGlyPheLeuIleAlaTyrGlnProLeuLeu      426
GGCCAGCCGCAGTACTACCTGGCCAATGGGGGCTTTCTGATCGCGTACCAGCCCCTTCTC    2159

SerAsnThrLeuAlaGluLeuTyrValArgGluHisLeuArgGluGlnSerArgLysPro      446
AGCAACACGCTCGCGGAGCTGTACGTGCGGGAACACCTCCGAGAGCAGAGCCGCAAGCCC    2219

ProAsnProThrProProProProGlyAlaSerAlaAsnAlaSerValGluArgIleLys      466
CCAAACCCCACGCCCCCGCCGCCCGGGGCCAGCGCCAACGCGTCCGTGGAGCGCATCAAG    2279

ThrThrSerSerIleGluPheAlaArgLeuGlnPhrThrTyrAsnHisIleGlnArgHis      486
ACCACCTCCTCCATCGAGTTCGCCCGGCTGCAGTTTACGTACAACCACATACAGCGCCAT    2339

ValAsnAspMetLeuGlyArgValAlaIleAlaTrpCysGluLeuGlnAsnHisGluLeu      506
GTCAACGATATGTTGGGCCGCGTTGCCATCGCGTGGTGCGAGCTGCAGAATCACGAGCTG    2399

ThrLeuTrpAsnGluAlaArgLysLeuAsnProAsnAlaIleAlaSerAlaThrValGly      526
ACCCTGTGGAACGAGGCCCGCAAGCTGAACCCCAACGCCATCGCCTCGGCCACCGTGGGC    2459

ArgArgValSerAlaArgMetLeuGlyAspValMetAlaValSerThrCysValProVal      546
CGGCGGGTGAGCGCGCGGATGCTCGGCGACGTGATGGCCGTCTCCACGTGCGTGCCGGTC    2519

AlaAlaAspAsnValIleValGlnAsnSerMetArgIleSerSerArgProGlyAlaCys      566
GCCGCGGACAACGTGATCGTCCAAAACTCGATGCGCATCAGCTCGCGGCCCGGGGCCTGC    2579

TyrSerArgProLeuValSerPheArgTyrGluAspGlnGlyProLeuValGluGlyGln      586
TACAGCCGCCCCCTGGTCAGCTTTCGGTACGAAGACCAGGGGCCCGTTGGTCGAGCGGCAG    2639
```

Fig. 6-3

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LeuGlyGluAsnAsnGluLeuArgLeuThrArgAspAlaIleGluProCysThrValGly      606
CTGGGGGAGAACAACGAGCTGCGGCTGACGCGCGATGCGATCGAGCCGTGCACCGTGGGA    2699

HisArgArgTyrPheThrPheGlyGlyGlyTyrValTyrPheGluGluTyrAlaTyrSer      626
CACCGGCGGCTACTTCACCTTCGGCGGGGGCTACGTGTACTTCGAGGAGTACGCGTACTCC    2759

HisGlnLeuSerArgAlaAspIleThrThrValSerThrPheIleAspLeuAsnIleThr      646
CACCAGCTGAGCCGCGCCGACATCACCACCGTCAGCACCTTCATCGACCTCAACATCACC    2819

MetLeuGluAspHisGluPheValProLeuGluValTyrThrArgHisGluIleLysAsp      666
ATGCTGGAGGATCACGAGTTTGTCCCCCTGGAGGTGTACACCCGCCACGCGATCAAGGAC    2879

SerGlyLeuLeuAspTyrThrGluValGlnArgArgAsnGluLeuHisAspLeuArgPhe      686
AGCGGCCTGCTGGACTACACGGAGGTCCAGCGCCGCAACCAGCTGCACGACCTGCGCTTC    2939

AlaAspIleAspThrValIleHisAlaAspAlaAsnAlaAlaMetPheAlaGlyLeuGly      706
GCCGACATCGACACGGTCATCCACGCCGACGCCAACGCCGCCATGTTCGCGGGCCTGGGC    2999

AlaPhePheGluGlyMetGlyAspLeuGlyArgAlaValGlyLysValValMetGlyIle      726
GCGTTCTTCGAGGGGATGGGCGACCTGGGGCGCGCGGTCGGCAAGGTGGTGATGGGCATC    3059

ValGlyGlyValValSerAlaValSerGlyValSerSerPheMetSerAsnProPheGly      746
GTGGGCGGCGTGGTATCGGCCGTGTCGGGCGTGTCCTCCTTCATGTCCAACCCCTTTGGG    3119

AlaLeuAlaValGlyLeuLeuValLeuAlaGlyLeuAlaAlaAlaPhePheAlaPheArg      766
GCGCTGGCCGTGGGTCTGTTGGTCCTGGCCGGCCTGGCGGCGGCTTTCTTCGCCTTTCGC    3179

TyrValMetArgLeuGlnSerAsnProMetLysAlaLeuTyrProLeuThrThrLysGlu      786
TACGTCATGCGGCTGCAGAGCAACCCCATGAAGGCCCTGTACCCGCTAACCACCAAGGAG    3239

LeuLysAsnProThrAsnProAspAlaSerGlyGluGlyGluGluGlyGlyAspPheAsp      806
CTCAAGAACCCCACCAACCCGGACGCGTCCGGGGAGGGCGAGGAGGGCGGCGACTTTGAC    3299

GluAlaLysLeuAlaGluAlaArgGluMetIleArgTyrMetAlaLeuValSerAlaMet      826
GAGGCCAAGCTAGCCGAGGCCCGGGAGATGATACGGTACATGGCCCTGGTGTCTGCCATG    3359

GluArgThrGluHisLysAlaLysLysLysGlyThrSerAlaLeuLeuSerAlaLysVal      846
GAGCGCACGGAACACAAGGCCAAGAAGAAGGGCACGAGCGCGCTGCTCAGCGCCAAGGTC    3419

ThrAspMetValMetArgLysArgArgAsnThrAsnTyrThrGlnValProAsnLysAsp      866
ACCGACATGGTCATGCGCAAGCGCCGCAACACCAACTACACCCAAGTTCCCAACAAAGAC    3479

                                                                 874
GlyAspAlaAspGluAspAspLeu
GGTGACGCCGACGAGGACGACCTGTGACGGGGGGGTTTGTTGTAAATAAAAACCACGGGTG

TTAAACCGCATGTGCATCTTTTGGTTTGTTTGTTTGGTACGCCTTTTGTGTGTGTGGGAA    3599

GAAAGAAAAGGGAACACATAAACTCCCCCGGGTGTCCGCGGCCTGTTTCCTCTTTCCTTT

CCCGTGACAAAACGGACCCCCTTGGTCAGTGCCGATTCCCCCCCCACGCCTTCCTCCACG    3719

TCGAAGGCTTTTGCATTGTAAAGCTACCCGCCTACCCGCGCCTCCCAATAAAAAAAGAAC

ATACACCAATGGGTCTTATTTGGTATTACCTGGTTTATTTAAAAAGATATACAGTAAGAC    3839

ATCCCATGGTACCAAAGACCGGGGCGAATCAGCGGGCCCCCATCATCTGAGAGACGAACA

AATCGGCGGCGCGGGCCGTGTCAACGTCCACGTGTGCTGCGCTGCTGGCGTTGACAAGGG    3959

CCCCGGCCTCCGCGTTGGATGCCTCCGGTTGGGATCC      3996
```

Fig. 7

```
 1
Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Gln Leu Gln
                    20
Leu Glu His Leu Leu Leu Asp Leu Gln Met Ile Leu Asn

Gly Ile Asn Asn Tyr Lys Asn Pro Lys Leu Thr Arg Met
40
Leu Thr Phe Lys Phe Tyr Met Pro Lys Lys Ala Thr Glu
                                60
Leu Lys His Leu Gln Cys Leu Glu Glu Glu Leu Lys Pro

Leu Glu Glu Val Leu Asn Leu Ala Gln Ser Lys Asn Phe
80
His Leu Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn Val
                                    100
Ile Val Leu Glu Leu Lys Gly Ser Glu Thr Thr Phe Met

Cys Glu Tyr Ala Asp Glu Thr Ala Thr Ile Val Glu Phe
120
Leu Asn Arg Trp Ile Thr Phe Cys Gln Ser Ile Ile Ser
133
Thr Leu Thr
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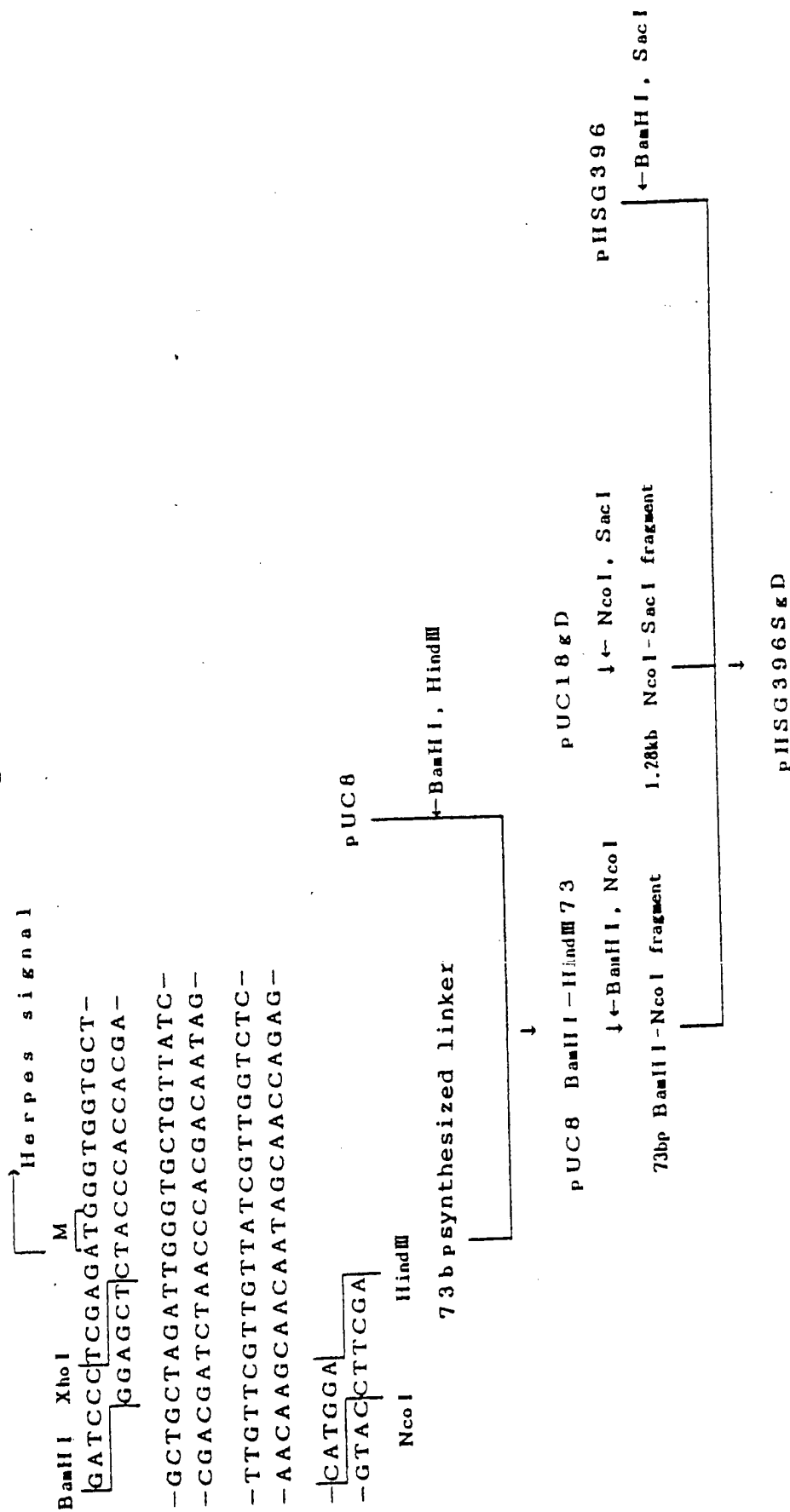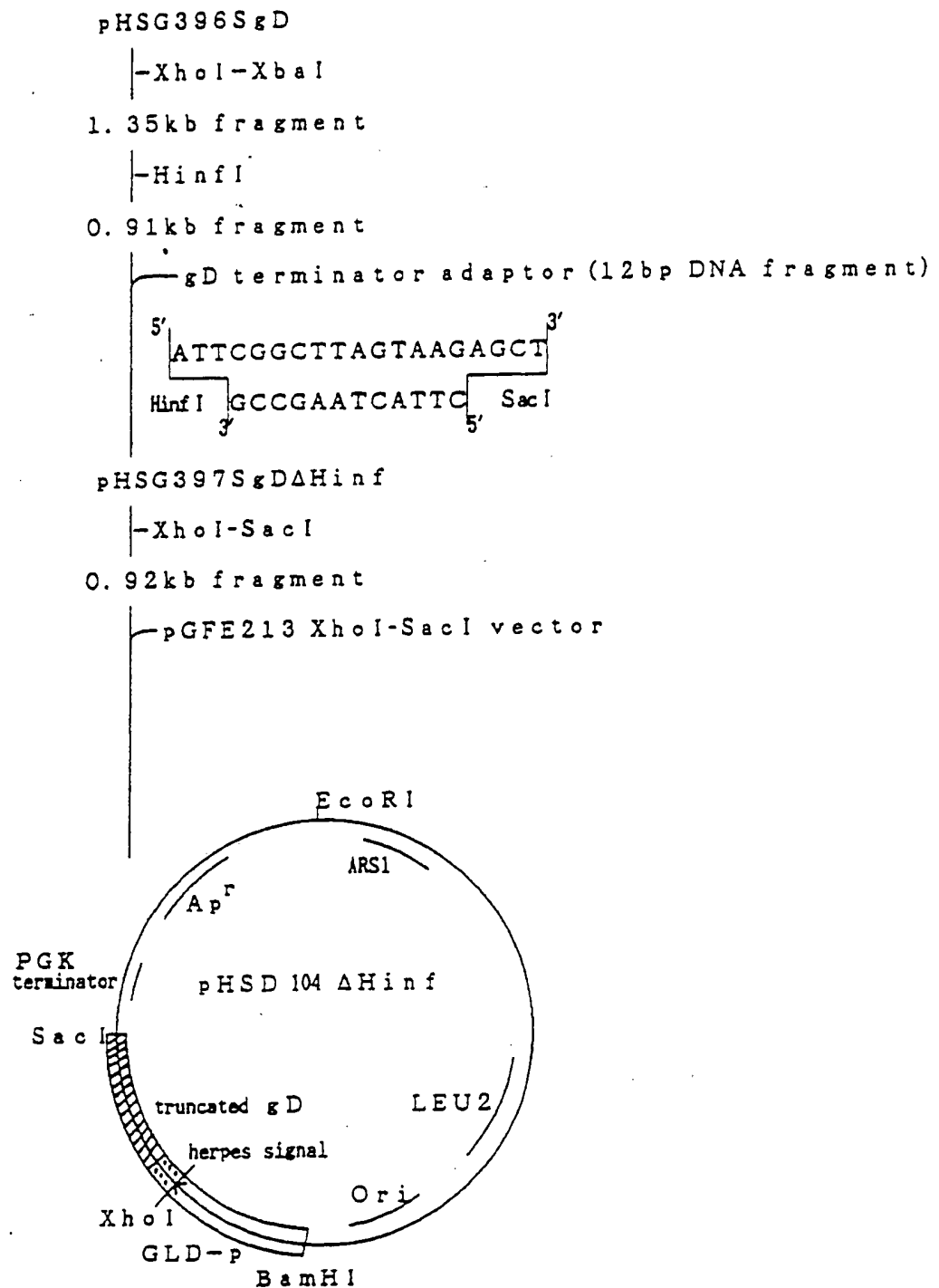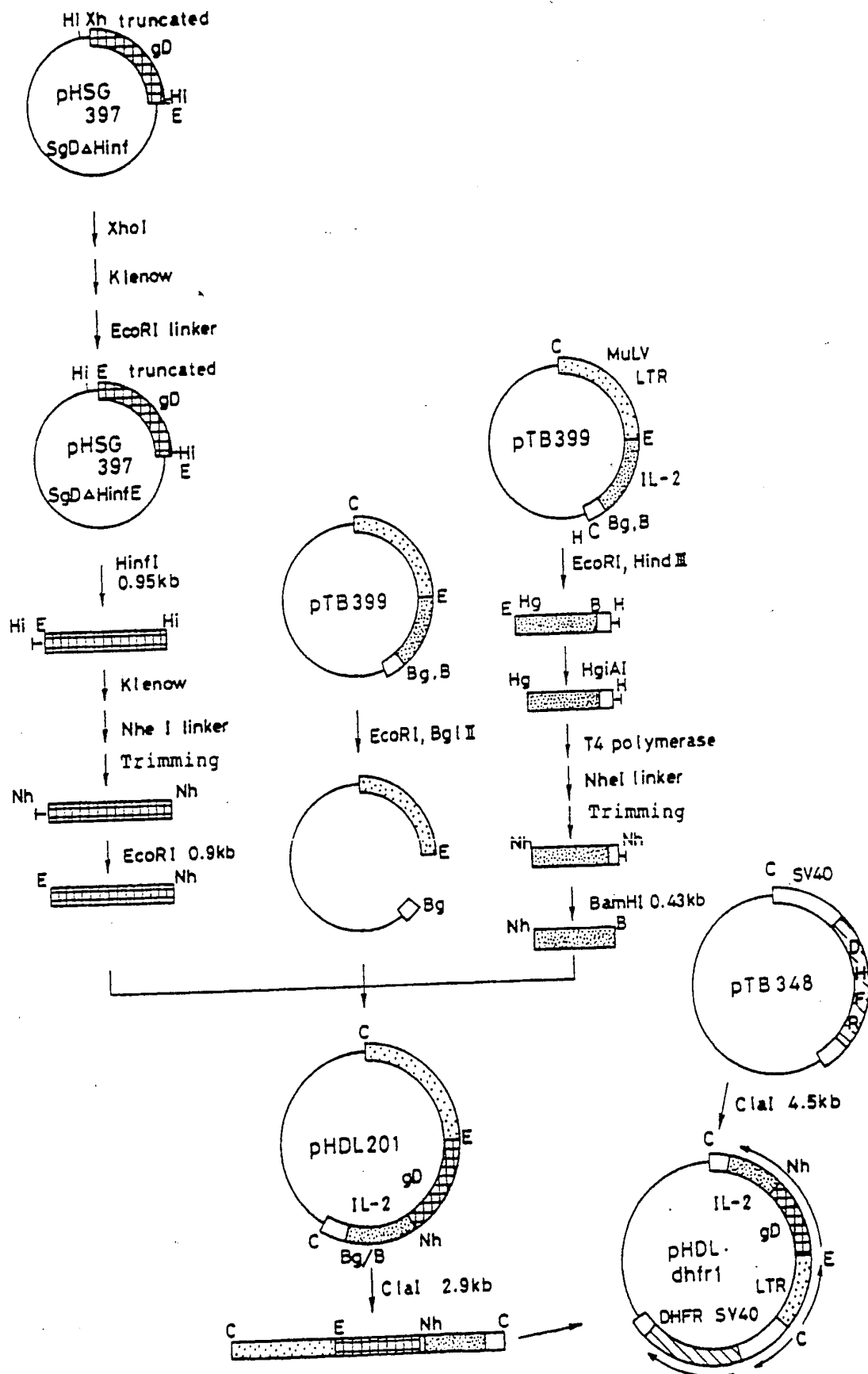
Fig. 8

Herpes signal

BamHI   XhoI   M
GATCCC|TCGAG|ATGGGTGGTGCT-
        |GGAGCT|CTACCCACCACGA-

-GCTGCTAGATTGGGTGCTGTTATC-
-CGACGATCTAACCCACGACAATAG-

-TTGTTCGTTGTTATCGTTGGTCTC-
-AACAAGCAACAATAGCAACCAGAG-

|CATGGA|
-|GTAC|CTTCGA|
  NcoI   HindIII

73 bp synthesized linker

pUC8
←BamHI, HindIII

pUC8 BamHI—HindIII 73
↓←BamHI, NcoI
73bp BamHI-NcoI fragment

pUC18gD
↓←NcoI, SacI
1.28kb NcoI-SacI fragment

pIISG396SgD

pIISG396
←BamHI, SacI

Fig. 9

pHSG396SgD

  ├-XhoI-XbaI

1. 35kb fragment

  ├-HinfI

0. 91kb fragment

  ├-gD terminator adaptor (12bp DNA fragment)

```
        5'                              3'
         ┌─────────────────────────┐
         │ATTCGGCTTAGTAAGAGCT       │
         └┐                        ┌┘
   HinfI   GCCGAATCATTC    SacI
          3'              5'
```

pHSG397SgDΔHinf

  ├-XhoI-SacI

0. 92kb fragment

  ├-pGFE213 XhoI-SacI vector

Fig. 10

Fig. 11

```
   1  ATG GGG GGG GCT GCC GCC AGG TTG GGG GCC GTG ATT TTG TTT GTC
  46  GTC ATA GTG GGC CTC CAT GGG GTC CGC GGC AAA TAT GCC TTG GCG
  91  GAT GCC TCT CTC AAG ATG GCC GAC CCC AAT CGC TTT CGC GGC AAA
 136  GAC CTT CCG GTC CTG GAC CCG CTG ACC GAC CCT CCG GGG GTC CGG
 181  CGC GTG TAC CAC ATC CAG GCG GGC CTA CCG GAC CCG TTC CAG CCC
 226  CCC AGC CTC CCG ATC ACG GTT TAC TAC GCC GTG TTG GAG CGC GCC
 271  TGC CGC AGC GTG CTC CTA AAC GCA CCG TCG GAG GCC CCC CAG ATT
 316  GTC CGC GGG GCC TCC GAA GAC GTC CGG AAA CAA CCC TAC AAC CTG
 361  ACC ATC GCT TGG TTT CGG ATG GGA GGC AAC TGT GCT ATC CCC ATC
 406  ACG GTC ATG GAG TAC ACC GAA TGC TCC TAC AAC AAG TCT CTG GGG
 451  GCC TGT CCC ATC CGA ACG CAG CCC CGC TGG AAC TAC TAT GAC AGC
 496  TTC AGC GCC GTC AGC GAG GAT AAC CTG GGG TTC CTG ATG CAC GCC
 541  CCC GCG TTT GAG ACC GCC GGC ACG TAC CTG CGG CTC GTG AAG ATA
 586  AAC GAC TGG ACG GAG ATT ACA CAG TTT ATC CTG GAG CAC CGA GCC
 631  AAG GGC TCC TGT AAG TAC GCC CTC CCG CTG CGC ATC CCC CCG TCA
 676  GCC TGC CTC TCC CCC CAG GCC TAC CAG CAG GGG GTG ACG GTG GAC
 721  AGC ATC GGG ATG CTG CCC CGC TTC ATC CCC GAG AAC CAG CGC ACC
·766  GTC GCC GTA TAC AGC TTG AAG ATC GCC GGG TGG CAC GGG CCC AAG
 811  GCC CCA TAC ACG AGC ACC CTG CTG CCC CCT GAG CTG TCC GAG ACC
 856  CCC AAC GCC ACG CAG CCA GAA CTC GCC CCG GAA GAC CCC GAG GAT
 901  TCG CTA GCG CCT ACT TCA AGT TCT ACA AAG AAA ACA CAG CTA CAA
 946  CTG GAG CAT TTA CTG CTG GAT TTA CAG ATG ATT TTG AAT GGA ATT
 991  AAT AAT TAC AAG AAT CCC AAA CTC ACC AGG ATG CTC ACA TTT AAG
1036  TTT TAC ATG CCC AAG AAG GCC ACA GAA CTG AAA CAT CTT CAG TGT
1081  CTA GAA GAA GAA CTC AAA CCT CTG GAG GAA GTG CTA AAT TTA GCT
1126  CAA AGC AAA AAC TTT CAC TTA AGA CCC AGG GAC TTA ATC AGC AAT
1171  ATC AAC GTA ATA GTT CTG GAA CTA AAG GGA TCT GAA ACA ACA TTC
1216  ATG TGT GAA TAT GCT GAT GAG ACA GCA ACC ATT GTA GAA TTT CTG
1261  AAC AGA TGG ATT ACC TTT TGT CAA AGC ATC ATC TCA ACA CTG ACT
1306  TGA
```

Fig. 12

```
  1  Met Gly Gly Ala Ala Ala Arg Leu Gly Ala Val Ile Leu Phe Val

 16  Val Ile Val Gly Leu His Gly Val Arg Gly Lys Tyr Ala Leu Ala

 31  Asp Ala Ser Leu Lys Met Ala Asp Pro Asn Arg Phe Arg Gly Lys

 46  Asp Leu Pro Val Leu Asp Pro Leu Thr Asp Pro Pro Gly Val Arg

 61  Arg Val Tyr His Ile Gln Ala Gly Leu Pro Asp Pro Phe Gln Pro

 76  Pro Ser Leu Pro Ile Thr Val Tyr Tyr Ala Val Leu Glu Arg Ala

 91  Cys Arg Ser Val Leu Leu Asn Ala Pro Ser Glu Ala Pro Gln Ile

106  Val Arg Gly Ala Ser Glu Asp Val Arg Lys Gln Pro Tyr Asn Leu

121  Thr Ile Ala Trp Phe Arg Met Gly Gly Asn Cys Ala Ile Pro Ile

136  Thr Val Met Glu Tyr Thr Glu Cys Ser Tyr Asn Lys Ser Leu Gly

151  Ala Cys Pro Ile Arg Thr Gln Pro Arg Trp Asn Tyr Tyr Asp Ser

166  Phe Ser Ala Val Ser Glu Asp Asn Leu Gly Phe Leu Met His Ala

181  Pro Ala Phe Glu Thr Ala Gly Thr Tyr Leu Arg Leu Val Lys Ile

196  Asn Asp Trp Thr Glu Ile Thr Gln Phe Ile Leu Glu His Arg Ala

211  Lys Gly Ser Cys Lys Tyr Ala Leu Pro Leu Arg Ile Pro Pro Ser

226  Ala Cys Leu Ser Pro Gln Ala Tyr Gln Gln Gly Val Thr Val Asp

241  Ser Ile Gly Met Leu Pro Arg Phe Ile Pro Glu Asn Gln Arg Thr

256  Val Ala Val Tyr Ser Leu Lys Ile Ala Gly Trp His Gly Pro Lys

271  Ala Pro Tyr Thr Ser Thr Leu Leu Pro Pro Glu Leu Ser Glu Thr

286  Pro Asn Ala Thr Gln Pro Glu Leu Ala Pro Glu Asp Pro Glu Asp

301  Ser Leu Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Gln Leu Gln

316  Leu Glu His Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile

331  Asn Asn Tyr Lys Asn Pro Lys Leu Thr Arg Met Leu Thr Phe Lys

346  Phe Tyr Met Pro Lys Lys Ala Thr Glu Leu Lys His Leu Gln Cys

361  Leu Glu Glu Glu Leu Lys Pro Leu Glu Glu Val Leu Asn Leu Ala

376  Gln Ser Lys Asn Phe His Leu Arg Pro Arg Asp Leu Ile Ser Asn

391  Ile Asn Val Ile Val Leu Glu Leu Lys Gly Ser Glu Thr Thr Phe

406  Met Cys Glu Tyr Ala Asp Glu Thr Ala Thr Ile Val Glu Phe Leu

421  Asn Arg Trp Ile Thr Phe Cys Gln Ser Ile Ile Ser Thr Leu Thr
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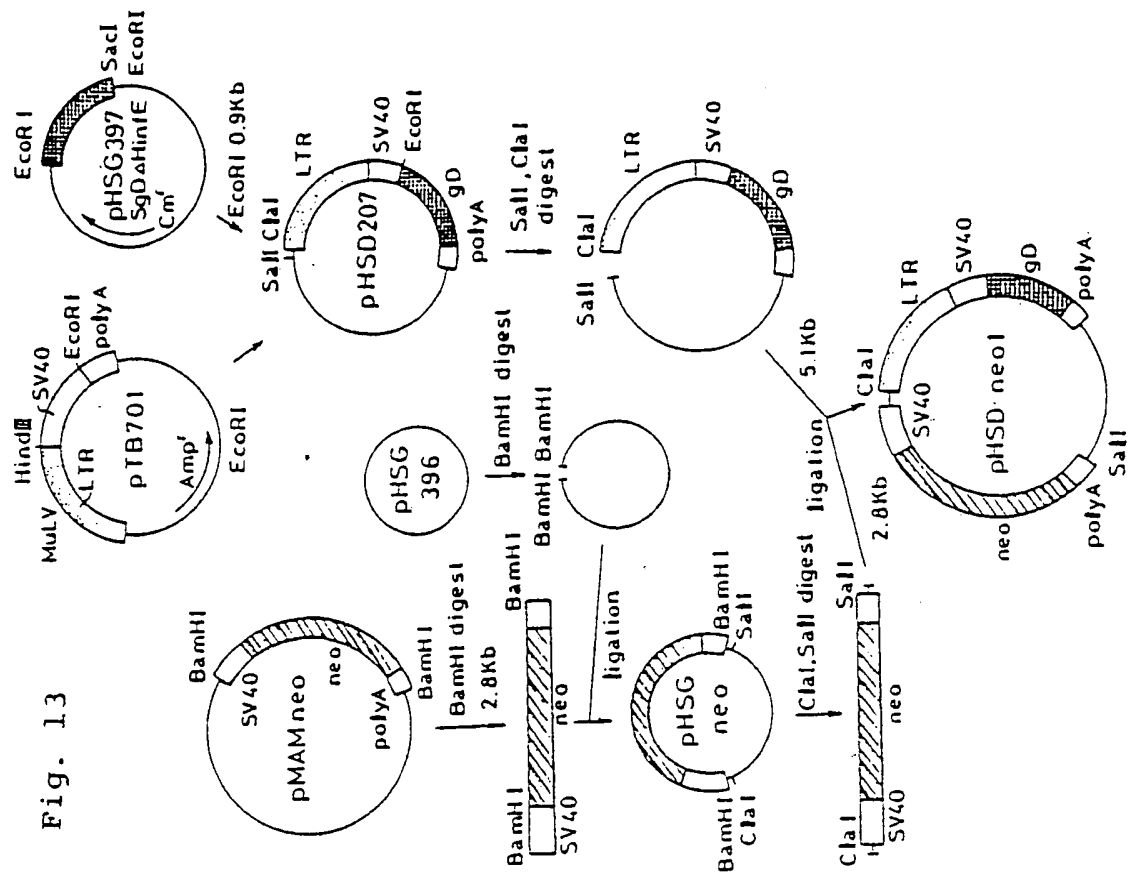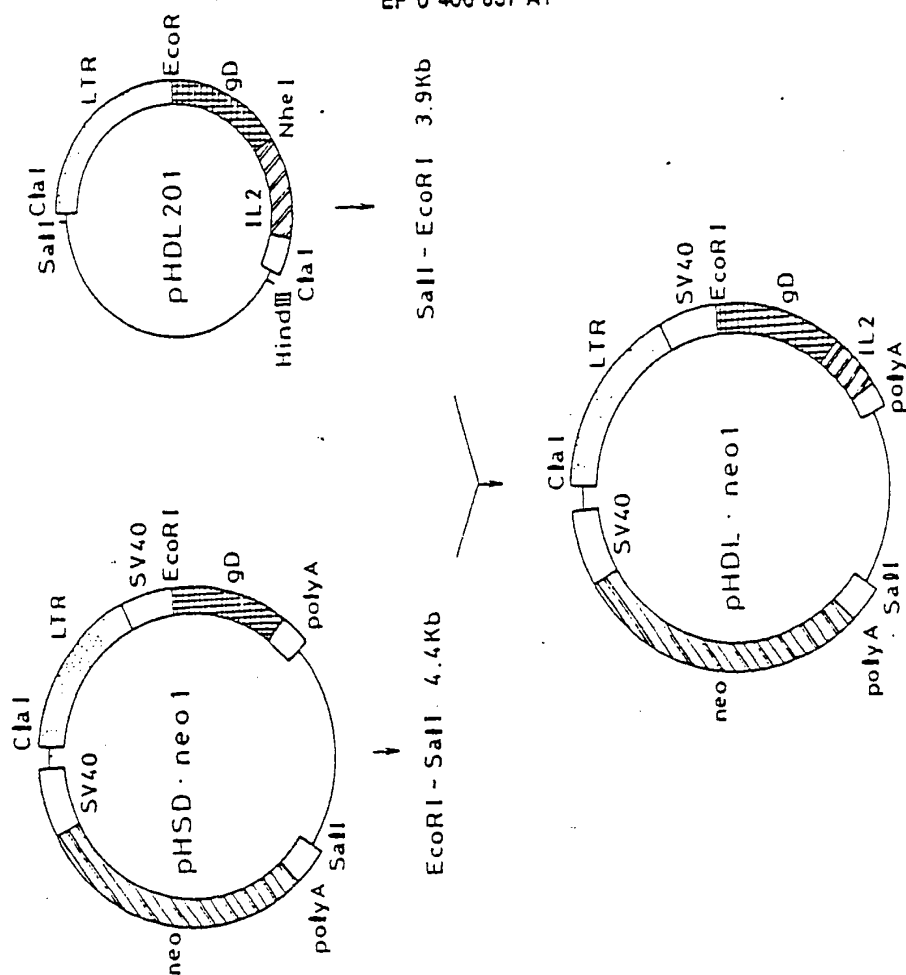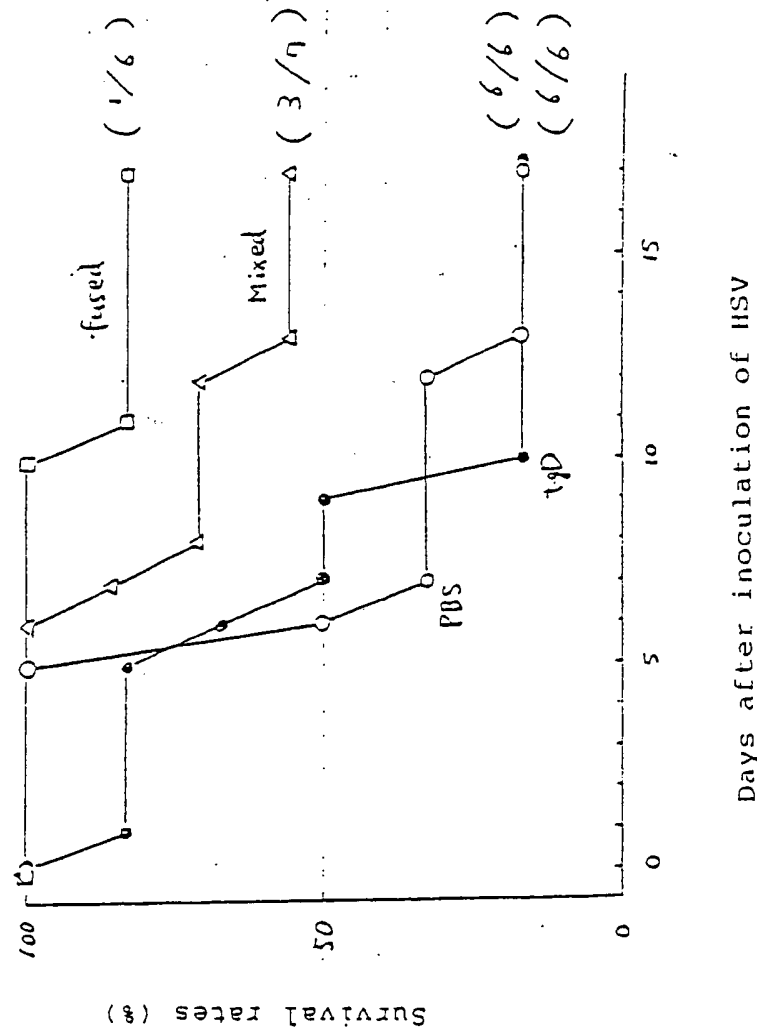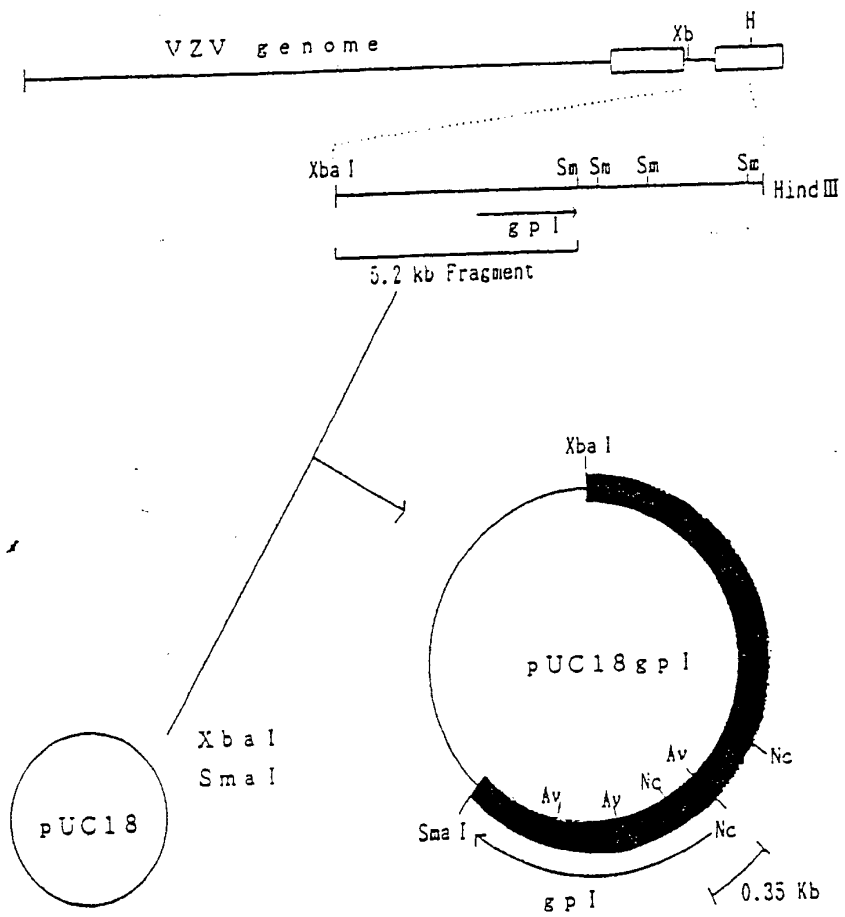
Fig. 13

Fig. 14

Fig. 15

Fig. 16-1

VZV genome

Xb
H

Xba I
Sm Sm Sm
Sm
Hind III

g p I

5.2 kb Fragment

Xba I
Sma I

pUC18

pUC18 g p I

Xba I

Av
Nc
Ay
Nc
Av
Sma I
Nc
g p I
0.35 Kb

Fig. 16-2

pUC18gp I
  |Nco I
  |Ava I

0.35 Kb Fragment

| Nco I
|BamH I   BamH I
|Ava I
|T4 DNA polymerase
|T4 DNA ligase

pUC19gp I Nco
    Xba I
    Klenow polymerase
    Kpn I
0.35 Kb Fragment

| EcoR I
| Klenow polymerase
| Kpn I

E/X

Kpn I

Nc

Nh

pUC18Nhegp I Nco

···GGATCCCCCCATGGGGGTACC···

BamH I   Nco I   Kpn I

pUC19Nco

···GGTACCCCTAGCTAGCTAGGGGGATCC···

Kpn I   Nhe I   BamH I

pUC18Nhe

Fig. 16-3

pUC18NhegpINco

   NheI

   Klenow Polymerase

   NcoI

1. 8 Kb

Fragment

pUC18gpISma

   EcoT221

   T4 DNA polymerase

NheI linker
5'-CTAGCTAGCTAG-3'

Fig. 16-4

pUC18NhegpIEcT

| XbaI

| Klenow polymerase

2.1 Kb Fragment

Sal I

MuLV LTR

Amp^r

pTB701

SV40 ori

EcoR I

poly A

EcoRI

Klenow polymerase

MuLV LTR

SV40 ori

E／X

Amp^r

Nc

t－gpI

pTBgpIEcT

Nhe I

E／X

Fig. 16-5

Fig. 16-6

pUC18gpISS60

E c o R I (partial)

5.5 Kb Fragment

Klenow polymerase

T4 DNA ligase

pUC18gpISS60-E7

X b a I

2.7 Kb Fragment
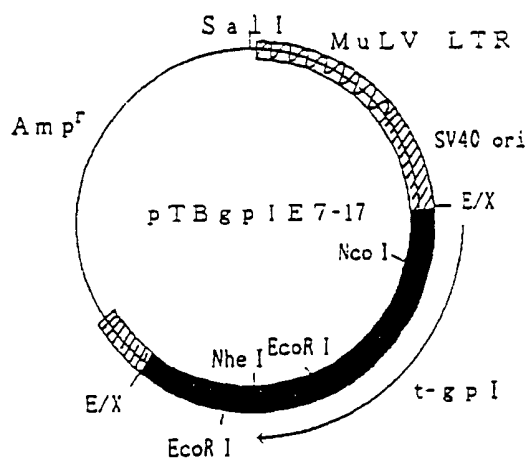
Klenow polymerase

──────────────── pTB701

E c o R I

Klenow polymerase

Sal I   MuLV LTR

Amp^r

SV40 ori

pTBgpIE7-17

E/X

Nco I

E/X   Nhe I   EcoR I

t-gpI

EcoR I

Fig. 16-7

pTBgpIE7-17
Sall
Klenow polymerase

Amp^r

C ASV LTR
SV40
hDHFR

1.9 Kb
Fragment

ClaI

pTB564

L

Klenow
polymerase

C

hDHFR
SV40
ASV LTR

Amp^r

MuLV LTR

pTBE7dhfr4

E/X

SV40 ori

E/X

t-gpI

Fig. 17—1

```
  1  CG AGA GCA GTC CAT GGT TTT AGA CCT CGG GCG AAT TGC GTG GTT TTA   47

                                            Met Gly Thr Val Asn Lys   6
 48  AGT GAC TAT ATT CCG AGG GTC GCC TGT AAT ATG GGG ACA GTT AAT AAA   95

  7  Pro Val Val Gly Val Leu Met Gly Phe Gly Ile Ile Thr Gly Thr Leu  22
 96  CCT GTG GTG GGG GTA TTG ATG GGG TTC GGA ATT ATC ACG GGA ACG TTG  143

 23  Arg Ile Thr Asn Pro Val Arg Ala Ser Val Leu Arg Tyr Asp Asp Phe  38
144  CGT ATA ACG AAT CCG GTC AGA GCA TCC GTC TTG CGA TAC GAT GAT TTT  191

 39  His Ile Asp Glu Asp Lys Leu Asp Thr Asn Ser Val Tyr Glu Pro Tyr  54
192  CAC ATC GAT GAA GAC AAA CTG GAT ACA AAC TCC GTA TAT GAG CCT TAC  239

 55  Tyr His Ser Asp His Ala Glu Ser Ser Trp Val Asn Arg Gly Glu Ser  70
240  TAC CAT TCA GAT CAT GCG GAG TCT TCA TGG GTA AAC CGG GGA GAG TCT  287

 71  Ser Arg Lys Ala Tyr Asp His Asn Ser Pro Tyr Ile Trp Pro Arg Asn  86
288  TCG CGA AAA GCG TAC GAT CAT AAC TCA CCT TAT ATA TGG CCA CGT AAT  335

 87  Asp Tyr Asp Gly Phe Leu Glu Asn Ala His Glu His His Gly Val Tyr 102
336  GAT TAT GAT GGA TTT TTA GAG AAC GCA CAC GAA CAC CAT GGG GTG TAT  383

103  Asn Gln Gly Arg Gly Ile Asp Ser Gly Glu Arg Leu Met Gln Pro Thr 118
384  AAT CAG GGC CGT GGT ATC GAT AGC GGG GAA CGG TTA ATG CAA CCC ACA  431

119  Gln Met Ser Ala Gln Glu Asp Leu Gly Asp Asp Thr Gly Ile His Val 134
432  CAA ATG TCT GCA CAG GAG GAT CTT GGG GAC GAT ACG GGC ATC CAC GTT  479

135  Ile Pro Thr Leu Asn Gly Asp Asp Arg His Lys Ile Val Asn Val Asp 150
480  ATC CCT ACG TTA AAC GGC GAT GAC AGA CAT AAA ATT GTA AAT GTG GAC  527

151  Gln Arg Gln Tyr Gly Asp Val Phe Lys Gly Asp Leu Asn Pro Lys Pro 166
528  CAA CGT CAA TAC GGT GAC GTG TTT AAA GGA GAT CTT AAT CCA AAA CCC  575

167  Gln Gly Gln Arg Leu Ile Glu Val Ser Val Glu Glu Asn His Pro Phe 182
576  CAA GGC CAA AGA CTC ATT GAG GTG TCA GTG GAA GAA AAT CAC CCG TTT  623

183  Thr Leu Arg Ala Pro Ile Gln Arg Ile Tyr Gly Val Arg Tyr Thr Glu 198
624  ACT TTA CGC GCA CCG ATT CAG CGG ATT TAT GGA GTC CGG TAC ACC GAG  671

199  Thr Trp Ser Phe Leu Pro Ser Leu Thr Cys Thr Gly Asp Ala Ala Pro 214
672  ACT TGG AGC TTT TTG CCG TCA TTA ACC TGT ACG GGA GAC GCA GCG CCC  719

215  Ala Ile Gln His Ile Cys Leu Lys His Thr Thr Cys Phe Gln Asp Val 230
720  GCC ATC CAG CAT ATA TGT TTA AAA CAT ACA ACA TGC TTT CAA GAC GTG  767

231  Val Val Asp Val Asp Cys Ala Glu Asn Thr Lys Glu Asp Gln Leu Ala 246
768  GTG GTG GAT GTG GAT TGC GCG GAA AAT ACT AAA GAG GAT CAG TTG GCC  815

247  Glu Ile Ser Tyr Arg Phe Gln Gly Lys Lys Glu Ala Asp Gln Pro Trp 262
816  GAA ATC AGT TAC CGT TTT CAA GGT AAG AAG GAA GCG GAC CAA CCG TGG  863

263  Ile Val Val Asn Thr Ser Thr Leu Phe Asp Glu Leu Glu Leu Asp Pro 278
864  ATT GTT GTA AAC ACG AGC ACA CTG TTT GAT GAA CTC GAA TTA GAC CCC  911

279  Pro Glu Ile Glu Pro Gly Val Leu Lys Val Leu Arg Thr Glu Lys Gln 294
912  CCC GAG ATT GAA CCG GGT GTC TTG AAA GTA CTT CGG ACA GAA AAA CAA  959

295  Tyr Leu Gly Val Tyr Ile Trp Asn Met Arg Gly Ser Asp Gly Thr Ser 310
960  TAC TTG GGT GTG TAC ATT TGG AAC ATG CGC GGC TCC GAT GGT ACG TCT 1007

311  Thr Tyr Ala Thr Phe Leu Val Thr Trp Lys Gly Asp Glu Lys Thr Arg 326
1008 ACC TAC GCC ACG TTT TTG GTC ACC TGG AAA GGG GAT GAA AAA ACA AGA 1055
```

Fig. 17-2

327 Asn Pro Thr Pro Ala Val Thr Pro Gln Pro Arg Gly Ala Glu Phe His 342
1056 AAC CCT ACG CCC GCA GTA ACT CCT CAA CCA AGA GGG GCT GAG TTT CAT 1103

343 Met Trp Asn Tyr His Ser His Val Phe Ser Val Gly Asp Thr Phe Ser 358
1104 ATG TGG AAT TAC CAC TCG CAT GTA TTT TCA GTT GGT GAT ACG TTT AGC 1151

359 Leu Ala Met His Leu Gln Tyr Lys Ile His Glu Ala Pro Phe Asp Leu 374
1152 TTG GCA ATG CAT CTT CAG TAT AAG ATA CAT GAA GCG CCA TTT GAT TTG 1199

375 Leu Leu Glu Trp Leu Tyr Val Pro Ile Asp Pro Thr Cys Gln Pro Met 390
1200 CTG TTA GAG TGG TTG TAT GTC CCC ATC GAT CCT ACA TGT CAA CCA ATG 1247

391 Arg Leu Tyr Ser Thr Cys Leu Tyr His Pro Asn Ala Pro Gln Cys Leu 406
1248 CGG TTA TAT TCT ACG TGT TTG TAT CAT CCC AAC GCA CCC CAA TGC CTC 1295

407 Ser His Met Asn Ser Gly Cys Thr Phe Thr Ser Pro His Leu Ala Gln 422
1296 TCT CAT ATG AAT TCC GGT TGT ACA TTT ACC TCG CCA CAT TTA GCC CAG 1343

423 Arg Val Ala Ser Thr Val Tyr Gln Asn Cys Glu His Ala Asp Asn Tyr 438
1344 CGT GTT GCA AGC ACA GTG TAT CAA AAT TGT GAA CAT GCA GAT AAC TAC 1391

439 Thr Ala Tyr Cys Leu Gly Ile Ser His Met Glu Pro Ser Phe Gly Leu 454
1392 ACC GCA TAT TGT CTG GGA ATA TCT CAT ATG GAG CCT AGC TTT GGT CTA 1439

455 Ile Leu His Asp Gly Gly Thr Thr Leu Lys Phe Val Asp Thr Pro Glu 470
1440 ATC TTA CAC GAC GGG GGC ACC ACG TTA AAG TTT GTA GAT ACA CCC GAG 1487

471 Ser Leu Ser Gly Leu Tyr Val Phe Val Val Tyr Phe Asn Gly His Val 486
1488 AGT TTG TCG GGA TTA TAC GTT TTT GTG GTG TAT TTT AAC GGG CAT GTT 1535

487 Glu Ala Val Ala Tyr Thr Val Val Ser Thr Val Asp His Phe Val Asn 502
1536 GAA GCC GTA GCA TAC ACT GTT GTA TCC ACA GTA GAT CAT TTT GTA AAC 1583

503 Ala Ile Glu Glu Arg Gly Phe Pro Pro Thr Ala Gly Gln Pro Pro Ala 518
1584 GCA ATT GAA GAG CGT GGA TTT CCG CCA ACG GCC GGT CAG CCA CCG GCG 1631

519 Thr Thr Lys Pro Lys Glu Ile Thr Pro Val Asn Pro Gly Thr Ser Pro 534
1632 ACT ACT AAA CCC AAG GAA ATT ACC CCC GTA AAC CCC GGA ACG TCA CCA 1679

535 Leu Leu Arg Tyr Ala Ala Trp Thr Gly Gly Leu Ala Ala Val Val Leu 550
1680 CTT CTA CGA TAT GCC GCA TGG ACC GGA GGG CTT GCA GCA GTA GTA CTT 1727

551 Leu Cys Leu Val Ile Phe Leu Ile Cys Thr Ala Lys Arg Met Arg Val 566
1728 TTA TGT CTC GTA ATA TTT TTA ATC TGT ACG GCT AAA CGA ATG AGG GTT 1775

567 Lys Ala Tyr Arg Val Asp Lys Ser Pro Tyr Asn Gln Ser Met Tyr Tyr 582
1776 AAA GCC TAT AGG GTA GAC AAG TCC CCG TAT AAC CAA AGC ATG TAT TAC 1823

583 Ala Gly Leu Pro Val Asp Asp Phe Glu Asp Ser Glu Ser Thr Asp Thr 598
1824 GCT GGC CTT CCA GTG GAC GAT TTC GAG GAC TCG GAA TCT ACG GAT ACG 1871

599 Glu Glu Glu Phe Gly Asn Ala Ile Gly Gly Ser His Gly Gly Ser Ser 614
1872 GAA GAA GAG TTT GGT AAC GCG ATT GGA GGG AGT CAC GGG GGT TCG AGT 1919

                                                                    624
615 Tyr Thr Val Tyr Ile Asp Lys Thr Arg ***
1920 TAC ACG GTG TAT ATA GAT AAG ACC CGG TGA TCA CCG AAC CGG GGC AAC 1967

1968 GTC GAG CGT GTA AAT TTA AAT AAA AAA CAG TAC GCT TTT ATC CGG TGT 2015

2016 ATG TTT TAA ATT TAT TTT TTT TTT TCT ATA TAA AGG GAT GGG GTG TCA 2063

2064 GGA TCT CTC GTA GGT TCT TGG GAC TCC AAG GGA CCC GCA GCC CAG GTA 2111

                                                                    2133
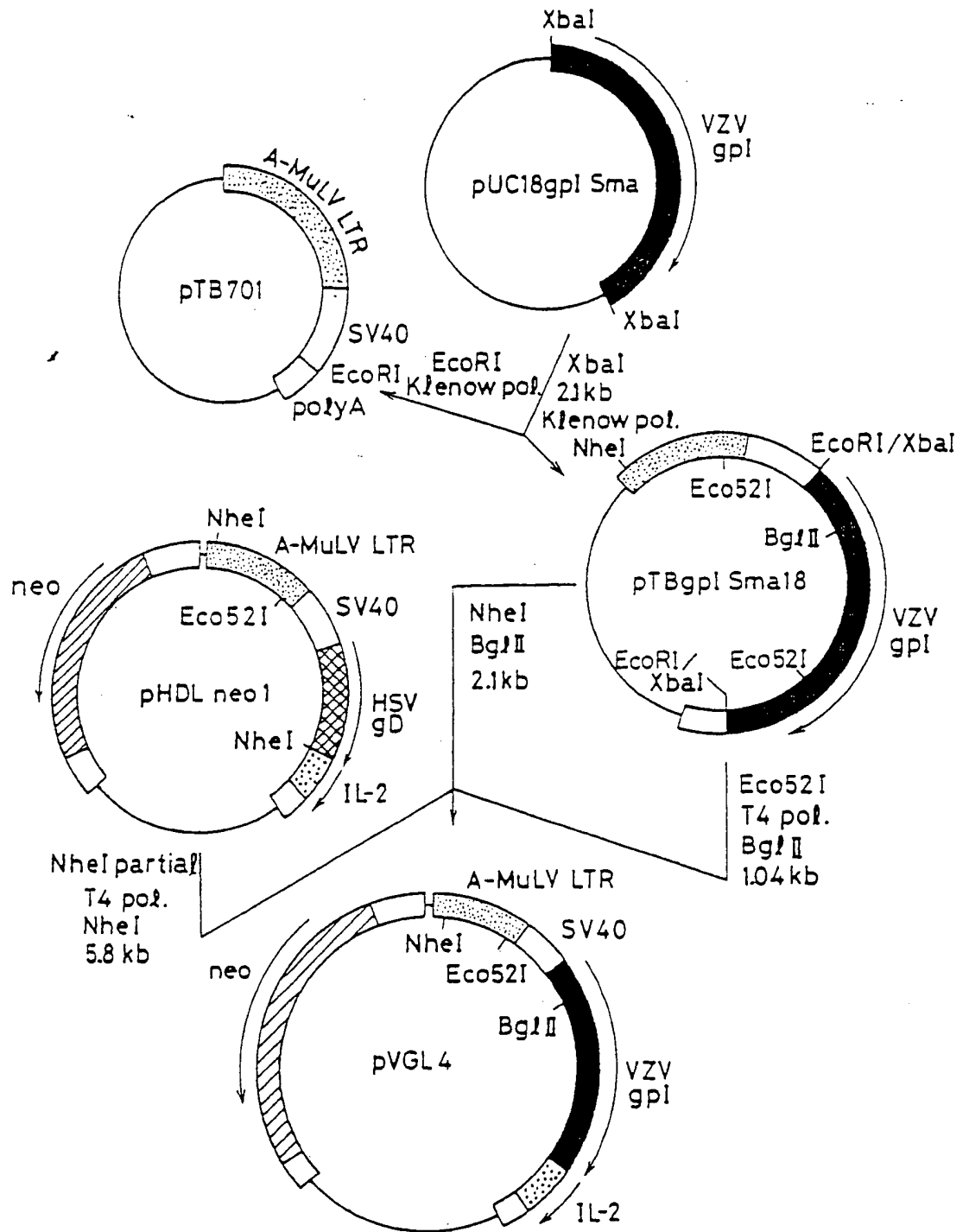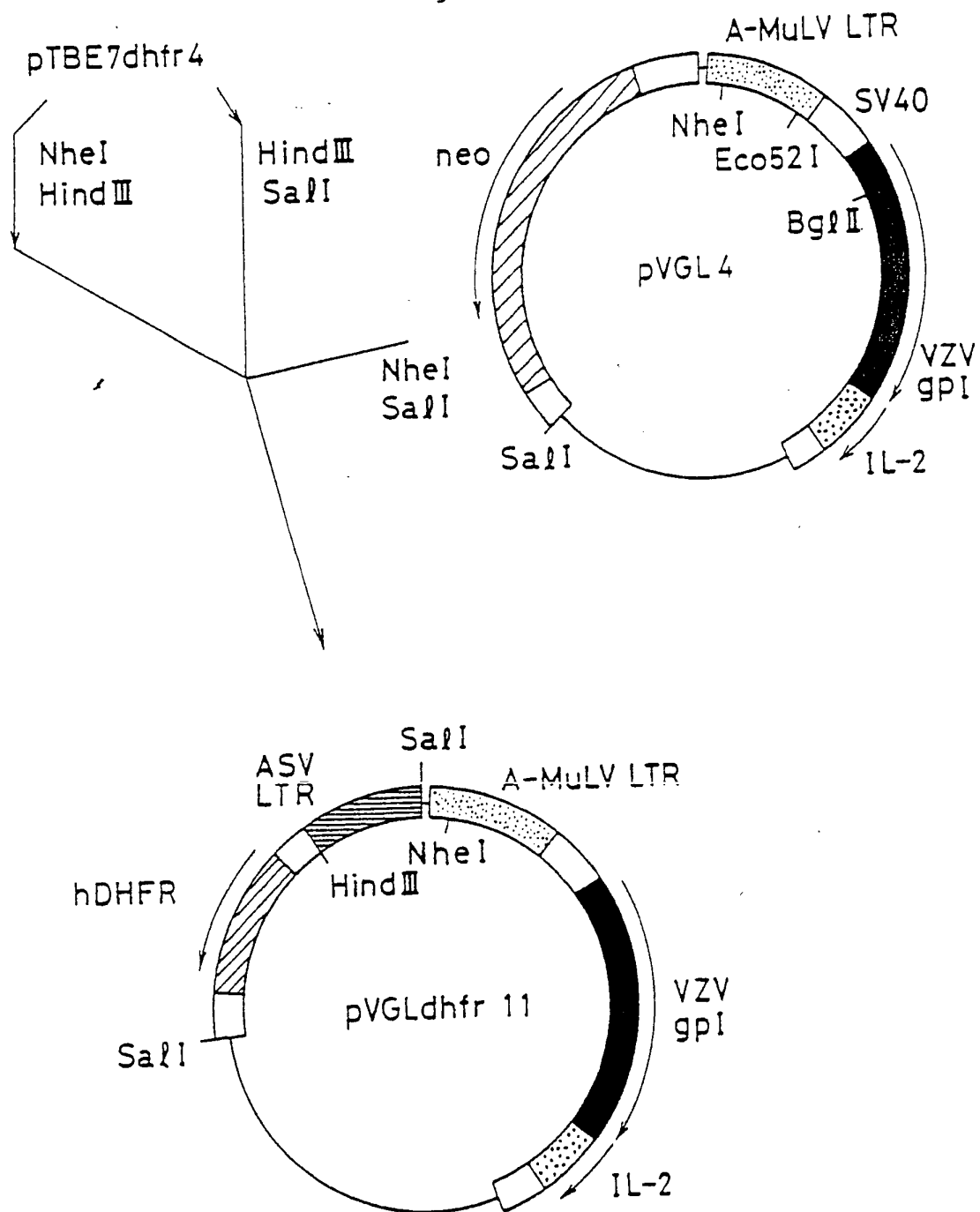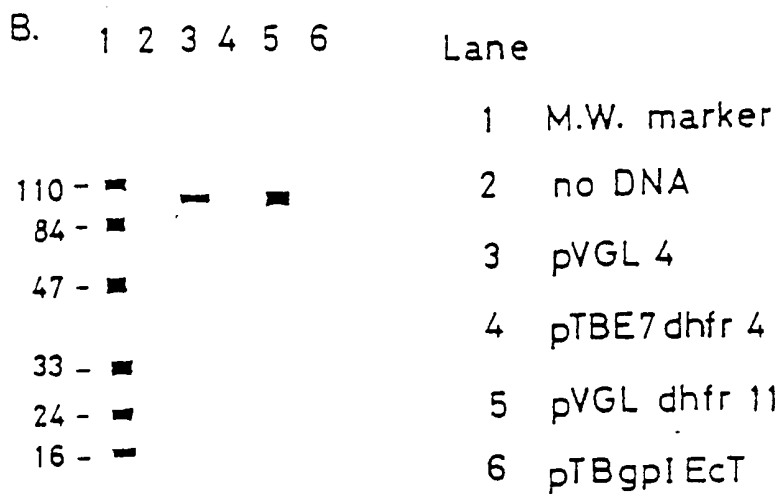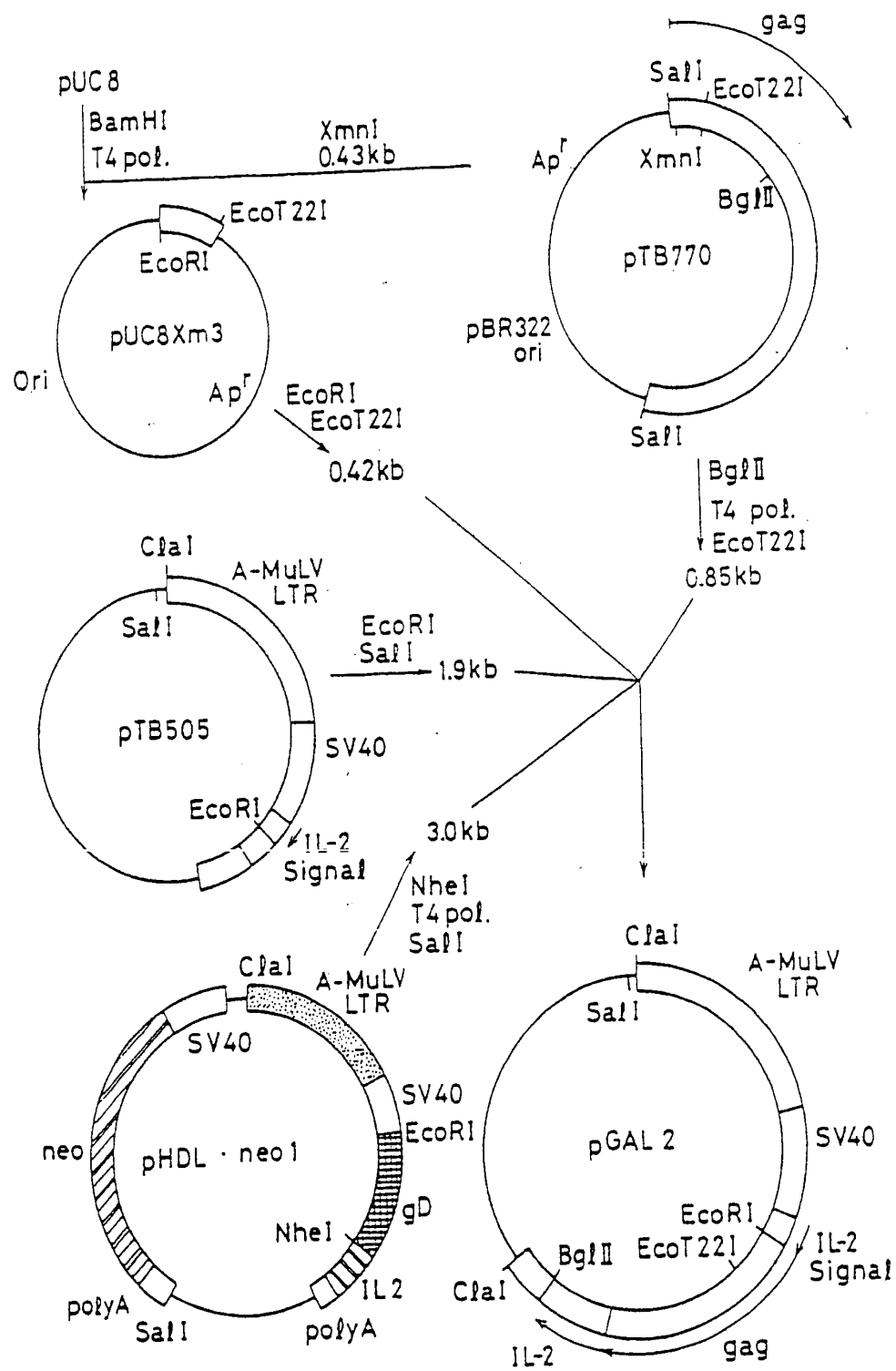2112 CGC GTC AAA AAG CCT GTG ACA A

Fig. 18

Fig. 19

pTBE7dhfr4

NheI
HindⅢ

HindⅢ
SaℓI

NheI
SaℓI

neo

A-MuLV LTR

NheI
Eco52I

SV40

BgℓⅡ

pVGL4

VZV
gpI

SaℓI

IL-2

SaℓI

ASV
LTR

A-MuLV LTR

hDHFR

HindⅢ

NheI

pVGLdhfr 11

VZV
gpI

SaℓI

IL-2

Fig. 20

A.

Lane

1 2 3 4 5 6

1 pTBgpI EcT

2 pVGL dhfr 11

3 pTBE7 dhfr 4

- 110   3 pTBE7 dhfr 4
- 84

4 pVGL 4

- 47

5 no DNA

- 33

6 M.W. marker

- 24
- 16

B.

1 2 3 4 5 6

Lane

1 M.W. marker

110 -

2 no DNA

84 -

47 -

3 pVGL 4

4 pTBE7 dhfr 4

33 -

24 -

5 pVGL dhfr 11

16 -

6 pTBgpI EcT

A.   anti-gpI

B.   anti-human IL-2

Fig. 21

Fig. 22

pHDL·neo 1   pGAL 2

ClaI   Bgl II   Bgl II

SalI   SalI   ClaI

2.8 kb   2.5 kb   3.6 kb

ClaI

neo

pGAL neo

EcoRI

gag

SalI

Bgl II

ClaI

IL-2

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	EP-A-150126 (IMMUNEX CORPORATION). * the whole document * ---	1-3, 26, 30	C12N15/62 C12N15/26 C12N15/38 C12N15/48 A61K39/21 A61K39/245 A61K37/24
A	WO-A-8901041 (GENENTECH, INC.) * pages 37 - 38, line 21; figure 3 * . ---	-	
A	WO-A-8800971 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) * page 3, lines 6 - 32 * * page 4, lines 5 - 26 * * page 6, line 11 - page 7, line 10; claims * -----	-	

TECHNICAL FIELDS
SEARCHED (Int. Cl.5)

C12N
C07K
A61K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	05 OCTOBER 1990	ANDRES S.M.